DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS FOR THE ESTIMATION OF ALVERINE CITRATE IN BULK AND IN PHARMACEUTICAL DOSAGE FORM BY UV SPECTROSCOPY AND RP-HPLC

Dissertation Submitted to

The Tamil Nadu Dr. M.G.R. Medical University, Chennai – 600 032.

In partial fulfillment for the award of degree of

MASTER OF PHARMACY

(Pharmaceutical Analysis)

Submitted by

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(Accredited by "NAAC" with CGPA of 2.74 on a four point scale at "B" Grade)

MELMARUVATHUR – 603 319

APRIL – 2014.

CERTIFICATE

This is to certify that the research work entitled **"DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS FOR THE ESTIMATION OF ALVERINE CITRATE IN BULK AND IN PHARMACEUTICAL DOSAGE FORM BY UV SPECTROSCOPY AND RP-HPLC"** submitted to The Tamil Nadu Dr. M.G.R. Medical University in partial fulfilment for the award of the degree of Master of Pharmacy (Pharmaceutical Analysis) was carried out by **JEETY SUNEEL KUMAR (RegisterNo:261230601)** in the Department of Pharmaceutical Analysis under my direct guidance and supervision during the academic year 2013-2014.

Place: Melmaruvathur

Date:

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ACKNOWLEDGEMENT

It gives me an immense pleasure to acknowledge, the help rendered to me by a host of a people, to whom I owe gratitude for successful completion of my Master of pharmacy (Pharmaceutical Analysis) degree course.

First and foremost, I wish to express my deep sense of gratitude to his Holiness **ARULTHIRU AMMA,** President, ACMEC Trust, Melmaruvathur for his ever growing blessings in each step of the study.

I am grateful to **THIRUMATHI LAKSHMI BANGARU ADIGALAR,** Vice President, ACMEC Trust, Melmaruvathur and **Dr.E.SRILEKHA,** correspondent, Adhiparasaakthi College of Pharmacy, Melmaruvathur for having given me an opportunity and encouragement all the way in completing the study.

The research work embodied in dissertation has been carried out under supervision of my esteemed and most respected guide **Dr. K.ANANDAKUMAR, M.Pharm., Ph.D.,** Associate Professor, Department of Pharmaceutical Analysis, Adhiparasakthi College of Pharmacy, my greatest debt of gratitude is to him for his continuous encouragement, valuable suggestions, dynamic guidance to elucidate problems and constant motivation throughout the dissertation work.

I take this opportunity to express my sincere thanks to our respected Principal **Prof. (Dr.) T. VETRICHELVAN, M.Pharm., Ph.D.,** Principal and Head, Department of Pharmaceutical Analysis, Adhiparasakthi College of Pharmacy, Melmaruvathur, for his constant support and encouragement. Without his supervision it would have been absolutely impossible to bring out the work in this manner.

I express my deep sense of gratitude to respected **Dr. D. NAGAVALLI, M.Pharm., Ph.D.,** Professor, **Mrs. G. ABIRAMI, M.Pharm.,** Assistant Professor and **Mr. J.SAMINATHAN, M.Pharm.,** Assistant Professor, Department of Pharmaceutical Analysis and other faculty members of Adhiparasakthi college of Pharmacy, Melmaruvathur, for their valuable help and guidance during the course of my research work.

I thank our laboratory staff members and librarian for their valuable support during the research work

I have great pleasure in express my sincere heartfelt thanks to **Mr. A.PRABANANTHAN, M.Pharm.,** for his support during this study.

Last but not least, with my whole heart, I would like to express my thanks to my father **Mr.J.M.MALLIKARJUNA**, my mother **Mrs. J. SUBHASHINI** and my brother **Mr. MANOJ KUMAR** for their encouragement and support which were a tower of strength during the entire course of work.

JETTY SUNEEL KUMAR

Dedicated to

My

Parents and

friends

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LIST OF ABBREVIATIONS USED

%	-	Percentage
% RSD	-	Percentage Relative Standard Deviation
μ	-	Micron
μl	-	Microlitre
$^{\circ}C$	-	Degree Celsius
gm	-	Grams
ICH	-	International Conference on Harmonisation
IR	-	Infra Red
LOD	-	Limit of Detection
LOQ	-	Limit of Quantitation
mg/ tab	-	Milligram Per tablet
min	-	Minute
ml	-	Millilitre
ml/ min	-	Millilitre/ Minute
nm	-	Nano meter
pH	-	Negative Logarithm of Hydrogen ion Concentration
rpm	-	Rotations Per Minute
Rt or t_R	-	Retention Time
SD	-	Standard Deviation
SE	-	Standard Error
IP	-	IndianPharmacopoeia
UV	-	Ultraviolet

v/v	-	Volume/ Volume
λ	-	Lambda
cm	-	Centimeter
µg/ ml	-	Microgram Per Millilitre
HPLC	-	High Performance Liquid Chromatography
DMF	-	Dimethyl Formamide
BP	-	British Pharmacopoeia
CI	-	Confidence Interval
µg	-	Micro gram

INTRODUCTION

1. INTRODUCTION

1.1 INTRODUCTION TO ANALYTICAL CHEMISTRY

(http://www.files.chem.vt.edu/chem-ed/crossref/ac-intro.html)

Analytical chemistry is the science of making quantitative measurements. In practice, quantifying analytes in a complex sample becomes an exercise in problem solving. To be effective and efficient, analyzing samples requires expertise in:

1. the chemistry that can occur in a sample
2. analysis and sample handling methods for a wide variety of problems (the tools-of-the-trade)
3. proper data analysis and record keeping

To meet these needs, Analytical Chemistry courses usually emphasize equilibrium, spectroscopic and electrochemical analysis, separations, and statistics. Analytical chemistry requires a broad background knowledge of chemical and physical concepts. These hypermedia documents contain links to the fundamental principles that underly the different analytical methods. With a fundamental understanding of analytical methods, a scientist faced with a difficult analytical problem can apply the most appropriate technique(s). A fundamental understanding also makes it easier to identify when a particular problem cannot be solved by traditional methods, and gives an analyst the knowledge that is needed to develop creative approaches or new analytical methods.

The methods of detecting analytes are the fallowing

1. Physical means
    - mass
    - colour
    - refractive index

- thermal conductivity

2. With electromagnetic radiation (Spectroscopy)
    - Absorption
    - Emission
    - Scattering
3. By an electric charge
    - Electrochemistry
    - Mass spectrometry

Thereare a limited number of ways to detect an analyte. However, in each of the above general categories there are a large multitude of specific analytical techniques.

1.1.1 Importance Of Analysis (*Kellner et al., 2004*)

Newer analytical methods are developed for the drugs or drug combinations due to the patient must receive the good quality of drugs, the drug or drug combinations may not be official in any Pharmacopoeia, a literature search may not reveal an analytical procedure for the drug or it combinations, analytical methods may not be available for the drug combinations due to interference caused by excipients and analytical methods for the quantification of drug or drug combinations from biological fluids may not be available.

The development of newer analytical methods are very importance in many of the fields like research institutions, quality control department in industries, approved testing laboratories, biopharmaceutical and bio equivalence study and clinical pharmacokinetic studies.

1.1.2 Disciplines Of Analytical Chemistry

(Gurdeep R. Chatwal et al., 2007; Napoleon, 2006; Kamboj, 2007)

There are two types of analysis. Those are as follows:

a) Qualitative analysis

b) Quantitative analysis

1.1.2.1 Qualitative analysis

This is practiced in order to establish the composition of naturally occur artificially synthesized or manufactured substances.

1.1.2.2 Quantitative analysis

There are several methods for quantitative analysis such methods are as mentioned below.

1.1.2.2.1 Chemical methods

1.1.2.2.1.1 Titrimetric analysis

The titrimetric methods are considered superior to gravimetric methods the analysis is similarly based on equivalent weight of one substance reacts quantitatively with the other.

i. Acid-base titrations

ii. Non-aqueous titrations

iii. Redox titrations

iv. Precipitation titrations

v. Complexometric titrations

1.1.2.2.1.2 Gravimetric analysis

The method involves the conversion of the element or a radical to be determined into a pure stable compound, readily convertible into a form suitable for weighing.

1.1.2.2.1.3 Gasometric analysis

The gases like cyclopropane, carbon dioxide, nitrous oxide, oxygen, octyl nitrate, nitrogen, amyl nitrate, ethylene and helium are determined by gasometric analysis. The measurement of volume of gas is usually done by means of gas burettes.

1.1.2.2.2 Physicochemical methods

These methods are mainly used for the measurement of certain physical properties and to determine the contents or composition of a substance. They are employed for the determination of trace concentrations of elements in preference to the sample. These are preferred due to their selectivity, simplicity, speed of analysis and accuracy than other methods. Most of the methods make use of standards having in the known amount of the constituents serves as the basis of comparison in the instrument. The changes in the properties of system detected by measurement of current, potential, electrical conductivity, specific rotation and refractive index.

1.1.2.2.3 Microbiological methods

Many microorganisms produce within themselves in chemical substances, which when excreted, interfere with the growth or metabolism of microorganisms. Such compounds are known as antibiotics and need to be present in low concentrations to bring out the antibiotic action. Antibiotics are chemotherapeutic agents. In micro biological methods comparison of the inhibition of the growth of the bacteria by a measured concentration of antibiotic which is to be examined is compared with the known concentration of the antibiotic standard preparation having known activity. Widely used methods are cup plate method and tube assay method.

1.1.2.2.4 Biological methods

When the potency of the drug or its derivative cannot be properly determined by physical or chemical methods, and where it is possible to observe the biological effects of the drug on some type of living matter, the biological assays are carried out on the basis of such assays is to determine how much of sample gives the same biological effect as a given quantity of the standard preparation. The sample and standard tested under identical conditions in all respect. In a typical bio-assay, a stimulus is applied to a subject. The intensity of stimulus applied to a subject is referred to as the dose and is indicated by a weight or in terms of the concentration of the preparation. The application of a stimulus on a subject produces some observable effect and this is called the response. The response may be measured by the total weight or weight of some organ of the subject, blood sugar concentration, and diameter of inhibition zone or by some other physiological symptoms.

1.2 SPECTROSCOPY*(http://www.wavesignal.com/forensics/Anlys.html)*

Spectroscopy is the study of matter and its properties by investigating light, sound, or particles that are emitted, absorbed or scattered by the matter under investigation. Spectroscopy may also be defined as the study of the interaction between light and matter. Historically, spectroscopy referred to a branch of science in which visible light was used for theoretical studies on the structure of matter and for qualitative and quantitative analyses. The definition has broadened, however, as new techniques have been developed that utilize not only visible light, but many other forms of electromagnetic and non-electromagnetic radiation including x-rays, microwaves, radio waves, electrons, phonons (sound waves) and others. Impedance spectroscopy is a study of frequency response in alternating current. Spectroscopy is often used for the identification of substances through the frequency spectrum emitted

or absorbed by them. A device for recording a spectrum is a spectrometer. Spectroscopy can be classified according to the physical quantity which is measured or calculated or the measurement process.

1.2.1 Spectroscopic Methods

(Gurudeep R. Chatwal, et al., 2008; Beckett and Stenlake, et al., 2007)

Spectroscopy deals with the interaction of an analyte with electromagnetic radiation. The interaction of the electromagnetic radiation results in absorption or emission radiations. Based on the absorption or emission the spectroscopy is classified into, absorption spectroscopy and emission spectroscopy.

1.2.1.1 Absorption spectroscopy

When a beam of electromagnetic radiation is passed through an analyte, certain amount of the radiation is absorbed into the matter. The analyte after absorbing the radiation goes from the ground state to the excited state giving the absorption spectra. The various absorption spectroscopies include the UV-Visible absorption,X-ray absorption, infrared absorption, microwave absorption, radio frequency absorption and atomic absorption, etc.

1.2.1.1.1 Atomic Absorption

In the atomic absorption spectra the electrons are excited from the lower energy state to a higher energy state by absorption of electromagnetic radiation. The atom absorbs the electromagnetic radiation of energy corresponding to the difference in energy between the higher and lower energy states of the absorbing atom. UV-Visible radiation can excite only the electrons in the outer most orbital, whereas the X-ray has the capacity to excite the electrons located in the inner shell near to the nuclei.

1.2.1.1.2 Molecular Absorption

The molecular absorption spectra of polyatomic molecules are more complex than the atomic absorption spectra since the number of energy states are higher. The energies associated with a molecule are rotational energy, vibrational energy and electronic energy.

$$E = E_{electronic} + E_{vibrational} + E_{rotational}$$

The molecule absorbs electromagnetic radiation of energy corresponding to the difference in the energy of the ground state molecule and the excited state molecule. The difference in energy ΔE is given by,

$$\Delta E = (E_{electronic} + E_{vibrational} + E_{rotational})_{excited} - (E_{electronic} + E_{vibrational} + E_{rotational})_{ground}$$

The UV-Visible radiations and X-rays have the energy to induce the transition from the ground state to the excited state.

1.2.1.2 Emission spectroscopy

Emission spectroscopy is the technique in which the wavelength of the photons emitted by an analyte due to the transition from higher energy level to lower energy on exposure to an electromagnetic radiation was studied. Each analyte emits a specific wavelength of radiation corresponding to the composition of the sample. The energy of the photons emitted is given by

$$E_{photon} = h\upsilon$$

Where E is the energy of the photon, ν is the frequency and h is the Planck's constant. The various instrumental techniques which are based on the measurement of the emitted radiation include Flame photometry, Fluorimetry, Radiochemical methods.

1.2.1.2.1 Atomic Emission

When an analyte is heated, it emits light characteristic of the atom present in it. For example, Sodium when heated emits yellow light and Potassium emits lilac light. When a metal is heated, the electrons in the outer orbital absorb the heat and goes to a higher energy state. The atom then comes back to the ground state by emitting the photons of light which has energy equal to the difference between the higher energy state and the lower energy state. The instrumental analytical technique, such as the flame emission spectroscopy works on the principle of measuring the photons of energy emitted by a thermally excited atom.

1.2.1.2.2 Molecular Emission

When a beam of electromagnetic radiation falls on a molecular species, it absorbs the radiation and gets excited. The excited molecules are short lived and it fall back to the ground state immediately. The molecules in the excited sate have higher vibrational energy than that of the ground state. They emit the absorbed energy by the following ways, fluorescence and phosphorescence.

The difference in the energy levels of the absorbed radiation, fluorescence and phosphorescence are as below

$$\Delta E_{absorption} > \Delta E_{fluorescence} > \Delta E_{phosphorescence}$$

The analytical techniques Spectrofluorimetry and Phosphorimetry involve the principle of molecular emission.

1.2.2 Ultraviolet Spectroscopy *(Beckett and Stenlake, 2002)*

This technique of ultra violet spectroscopy is one of most frequently employed method in pharmaceutical analysis. It involves the measurement of the amount of UV radiation (190 - 380 nm) or visible (380 - 800 nm) radiation absorbed by a substance in solution. Ultraviolet spectroscopy involves the promotion of electrons (σ, π, n electrons)

from the ground state to higher energy state. It is useful to measure the number of conjugated double bonds and also aromatic conjugation with the various molecules.

The ultraviolet region of the electromagnetic spectrum is frequently subdivided into as follows:

- Far vacuum Ultraviolet region (10 - 200 nm)
- Near Ultraviolet region (200 - 400 nm)
- Visible region (380 - 780 nm)

Ultra violet absorption spectra are attributed to a process in which the outer electron of atoms or molecules absorbs radiant energy and undergoes transitions to higher energy level. Their transitions arequantitized and depend on electronic structure of the absorbent. Many molecules absorb ultraviolet or visible light. The absorbance of a solution increases as attenuation of the beam increases. Absorbance is directly proportional to the path length, *b*, and the concentration, *c*, of the absorbing species. Beer's Law states that

$$A = \varepsilon bc,$$

Where,

ε Is a constant of proportionality, called the absorptivity.

Different molecules absorb radiation of different wavelengths. An absorption spectrum will show a number of absorption bands corresponding to structural groups within the molecule.

1.2.2.1 Beer - Lambert's Law

(B.K Sharma, 2006; Robert D. Braun et al., 2006; Gurudeep R. Chatwal et al, 2008)

When light is incident upon a homogeneous medium, a part of incident light is reflected, a part is absorbed by the medium and the remainder is allowed to transmit as such.

$I_0 = I_a + I_t + I_r$

Where,

I_0 = Incident light

I_a = Absorbed light

I_t = Transmitted light

I_r = Reflected light

Lambert's Law states "when a beam of light is allowed to pass through a transparent medium, the rate of decrease of intensity with the thickness of the medium is directly proportional to the intensity of the light".

$$-\frac{dI}{dt} \propto I$$

$I_t = I_0 e^{-kt}$---------- (1)

Beer's Law states "The intensity of a beam of monochromatic light decreases exponentially with the increase in concentration of the absorbing substance".

$$-\frac{dI}{dc} \propto I$$

$I_t = I_0 e^{-k'c}$---------- (2)

(or)

$$In\frac{I_0}{I_t} = -k'c$$

By solving equations 1 and 2, on changing equations from natural logarithm,

$I_t = I_0 . 10^{-0.4343kt} = I_0 10^{-kt}$---------- (3)

$I_t = I_0 . 10^{-0.4343 k'c} = I_0^{-k'c}$ ---------- (4)

On combining equations 3 and 4,

$$I_t = I_0 10^{-act}$$

$$\log\frac{I_0}{I_t} = act$$

Where k and k' are constants, C is the concentration of the absorbing substance and t denotes thickness of the medium.

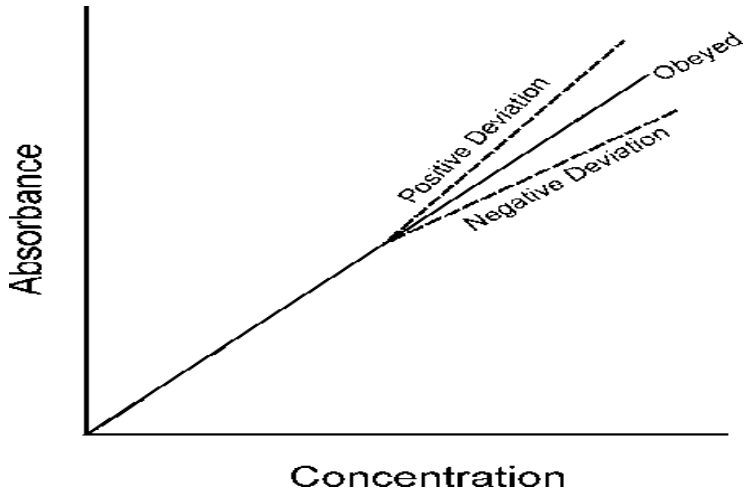
1.2.2.1.1 Limitations of Beer's law

The linearity of the Beer-Lambert law is limited by chemical and instrumental factors. Causes of nonlinearity include:

- Deviations in absorptivity coefficients at high concentrations (>0.01M) due to electrostatic interactions between molecules in close proximity.
- Scattering of light due to particulates in the sample.
- Fluorescence or phosphorescence of the sample.
- Changes in refractive index at high analyte concentration.
- Shifts in chemical equilibrium as a function of concentration.
- Non-monochromatic radiation, deviations can be minimized by using a relatively flat part of the absorption spectrum such as the maximum of an absorption band.
- Stray light.

1.2.2.1.2 Deviations from Beer's law

According to Beer's law, a straight line passing through the origin should be obtained, when a graph is plotted between absorbance and concentration. But there is always a deviation from linear relationship between absorbance and concentration and intact the shape of an absorption curve usually changes with changes in concentration of solution and unless precautions are observed. Deviations from the law may be positive or negative according to whether the resulting curve is concave upward or concave downward.



The latter two are generally known as instrumental deviation and chemical deviation.

a. *Instrumental deviations*

Stray radiation, Improper slit width, Fluctuation in single beam.

b. *Chemical deviations*

Hydrolysis, Association, Polymerization, Ionization and Hydrogen bonding

Deviations from Beer's law can arise due to the following factors

1. Beer's law will hold over a wide range of concentration provided the structure of coloured ion or of the coloured non electrolyte in the dissolved state does not change with concentration. If a coloured solution is having a foreign substance whose ions do not react chemically with the coloured components, its small concentration does not affect the light absorption and may also alter the value of extinction co - efficient.
2. Deviations may also occur if the coloured solute ions dissociates or associates.
3. Deviations may also occur due to the presence of impurities that fluorescence or absorb at absorption wavelength.
4. Deviations may occur if monochromatic light is not used.

5. Deviations may occur if the width of slit is not proper and therefore it allows undesirable radiations to fall on the detector.
6. Deviations may occur if the solution undergoes polymerization.
7. Beer's law cannot apply to suspensions but the latter can estimated calorimetrically after preparing a reference curve with known concentrations.

1.2.2.2 Choice of solvent (*William Kemp, 2006)*

A suitable solvent for ultraviolet spectroscopy should meet the following requirements.

(i) It should not itself absorb radiations in the region under investigation.

(ii) It should be less Polar so that it has minimum interaction with the solute molecule.

Solvents used in UV spectroscopy.

S.No.	Solvent	Cut-off (nm)
1.	Ethanol	205
2.	Methanol	210
3.	Acetonitrile	210
4.	Hexane	210
5.	Cyclohexane	210
6.	Diethyl ether	220
7.	Chloroform	245
8.	Carbon tetrachloride	265

1.2.2.3 Transitions in organic molecules

(Sharma Y.R, et al., 2009; Gurdeep R. Chatwal, et al., 2008)

The absorption in the ultraviolet region results in the transition of the valence electron form the ground level to the excited level. The three types of electron transitions are

Sigma (σ) electrons: These are involved in the formation of saturated bonds. The energy required for the excitation of the electrons is more than that of the UV radiations. Hence these electrons do not absorb near UV radiation.

Pi (π) electrons: These are involved in the formation of unsaturated bonds.

Example: Dienes, trienes and aromatic compounds. It absorbs radiation in near UV region.

Nonbonding (n) electrons*:* These are the lone pair of electrons present in atoms such as oxygen, nitrogen etc., in a molecule. They can be excited by both UV and Visible radiations.

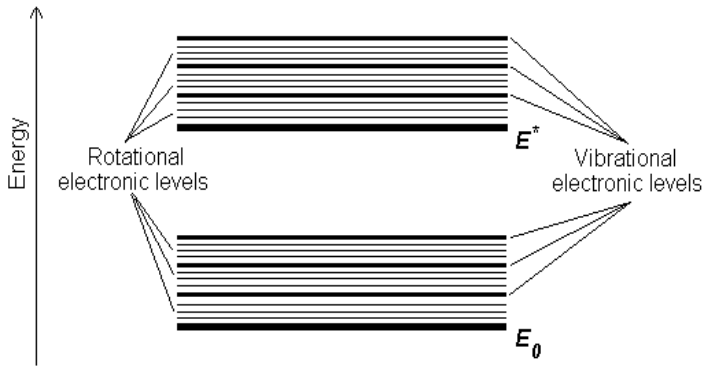
The various types of transitions are

$\sigma \rightarrow \sigma^*$

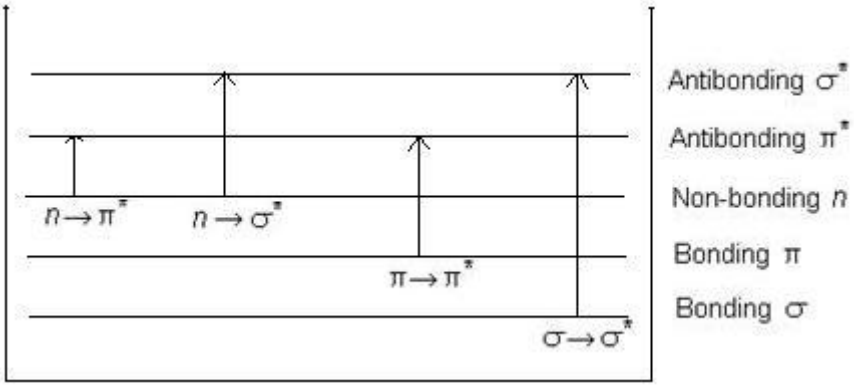
$n \rightarrow \sigma^*$

$\pi \rightarrow \pi^*$

$n \rightarrow \pi^*$



(http://teaching.shu.ac.uk/hwb/chemistry/tutorials/molspec/uvvisab1.html)



(http://www.pharmatutor.org/pharma-analysis/analytical-aspects-of-uv-visible-spectroscopy/types-of-electrone-transition.html)

The energy required for the various types of transitions are

σ→σ* >n→σ* >π → π * > n→ π *

σ→σ* transitions

These transitions occur in saturated hydrocarbons with single bonds and no lone pair of electrons. The energy required for this type of transition is very high because of the strong sigma bond formed by the valence electrons. Thus, the transitions occur at very short wavelength. The saturated hydrocarbons such as methane, ethane, propane etc. absorbs at 126 -135 nm region of the UV region. Hence these compounds are used as solvents in UV spectroscopy.

n → σ* transitions

Saturated compounds with lone pair of electrons show n→σ* transitions in addition to σ→σ* transitions. The energy required for the n→σ* transition is lesser than the energy required for σ→σ* transitions. The energy required for n→σ* transition, in alkyl halides, decreases with increase in the size of the halogen atom. Alcohols and amines forms hydrogen bonding with the solvent hence require higher energy for the transitions.

π → π * transitions

These transitions occur in unsaturated compounds containing double or triple bonds and also in aromatic compounds. Lower energy is required for these transitions and hence a longer wavelength causes the excitation of the molecule.

n→ π* transitions

These transitions occur in compound which contains oxygen, nitrogen, sulphur and halogens because of the presence of free lone pair of electrons. These transitions require least amount of energy and hence they occur in UV and Visible region. Saturated carbonyl compounds shows two types of transitions, low energy n→ π^* transitions occurring at longer wavelength and high energy n→ π^* transitions occurring at lower wavelength. The shifts in the absorption of the carbonyl compounds are due to the polarity of the solvent.

1.2.2.4 Instrumentation*(Gurudeep R. Chatwal et al., 2008)*

(*http://www2.chemistry.msu.edu/faculty/reusch/virtTxtJml/Spectrpy/UVVis/uvspec.html)*

All photometers, colorimeters and spectrophotometers have the following basic components

1.2.2.4.1 Radiation source

i) It must be stable.

ii) It must be of sufficient intensity for the transmitted energy to be detected at the end of the optical path.

iii) It must supply continuous radiation over the entire wavelength region in which it is used.

UV region

Hydrogen discharge lamp, Deuterium discharge lamp, Xenon arc Lamp.

Visible region

The tungsten lamp and tungsten halogen lamp are the most common source of visible radiation.

1.2.2.4.2 Filters and Monochromators

The filters and Monochromators are used to disperse the radiation according to the wavelength.

1.2.2.4.2.1 Filters

A light filter is a device that allows light of the required wavelength to pass but absorbs light of other wavelengths wholly or partially. Thus, a suitable filter can be selecting a desired wavelength band. It means that a particular filter may be used for a specific analysis. If analysis is carried out for several species, a large number of filters have to be used and interchanged. This method is very useful for routine analysis.

Types of filters

Filters are two types,

i) Absorption filters

ii) Interference filters

Absorption filters work by selective absorption of unwanted wavelengths and are made up of solid sheet of glass, coloured by a pigment or dispersed in glass and dyed gelatin. Interference filters work by selective transmission of selected wavelengths and they are made up of semitransparent metal film deposited on a glass plate and coated with dielectric material.

1.2.2.4.2.2 Monochromators

Monochromators successfully isolates band of wavelengths usually much more than a narrower filter. The essential elements of a Monochromators are an

entrance slit, a dispersing element (prim or gratings) and an exit slit. The entrance slit sharply defines the incoming beam of heterochromatic radiation. The dispersing element disperses the heterochromatic radiation into its component wavelengths whereas exit slit allows the nominal wavelength together with a band of wavelength on either side of it. The position of the dispersing element is always adjusted by rotating it to vary the nominal wavelength passing through the exit slit.

Types of Monochromators

1) Prisms

2) Gratings

A prism is made up of quartz (for UV region), glass (for visual range) and alkali halides (for IR). The main advantage of prisms is that they undergo dispersion giving wavelengths which do not overlap, but the main disadvantage is that they give non – linear dispersion. A grating consists of large number of parallel lines ruled on a highly polished surface like alumina. Generally, gratings are difficult to prepare therefore, replica gratings are prepared from an original grating. This is done by coating the original grating with a film of an epoxy resin which after setting is removed to yield replica. Then replica is made reflective by aluminizing its surface. Gratings give linear dispersion but they suffer from an overlap of spectral orders.

1.2.2.4.3 Sample cells

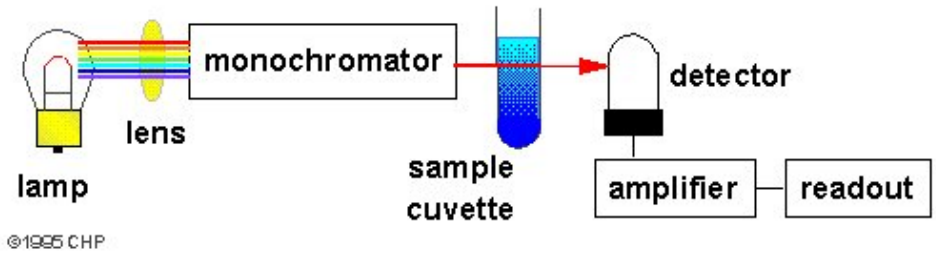
These are containers for holding the sample and reference solutions and must be transparent to the radiation passing through generally with a thickness of 1 Cm. The choice of a sample cells are based on transmission characteristics at desired wave lengths, the path length, shape, size and the relative expense. The transmission characteristics are based on the construction materials. For UV region, the cells made up of quartz and for visible region, the cells are made of glass.

1.2.2.4.4 Detectors

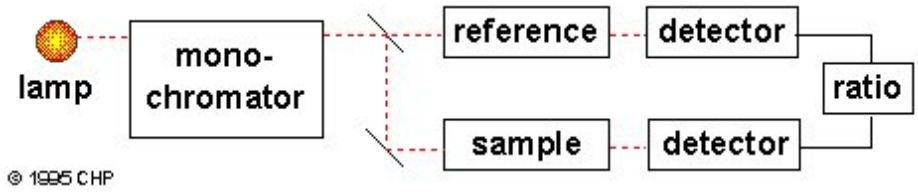
Detectors used in UV-Visible spectrophotometers can be called as photometric detectors. In these detectors the light energy is converted to electrical signal which can be recorded. The types of detectors used are Barrier Layer cell (or) Photo Voltaic cell, Photo tubes (or) Photo emissive tubes, Photomultiplier tubes and Photo diode.

1.2.2.4.5 Recorders

Detectors transmits the amount of light absorbed by a particular chemical species and only by that species is desired and by correcting the absorbance of solvent and other species in the solution. The recorders record the spectrum without any interferences compared with blank and they are user friendly.



Schematic diagram of a single beam UV-Vis spectrophotometer



Schematic diagram of a dual-beam uv-vis spectrophotometer

(http://www.chemistry.adelaide.edu.au/external/soc-rel/content/uv-vis.html)

Absorption spectroscopy is one of the most useful and widely used tools available to the analyst for quantitative analysis. The relation between the concentration of analyte and the amount of light absorbed is the basis of most analytical applications of molecular spectroscopy. This method of analysis is gaining importance due to simple,

rapid, precise, spectra of highly accurate and less time consuming. Spectrophotometric multi-component analysis can be applied where the drugs overlaps. In such cases of overlapping spectra, simultaneous equation can be framed to obtain the concentration of individual component otherwise multi-component analysis can be applied on any degree of spectral overlap provided that two or more spectra are not similar. The various spectroscopic techniques used for multi component analysis include

1. Simultaneous equation method
2. Absorption ratio method
3. Geometric correction method
4. Absorption correction method
5. Orthogonal polynomial method
6. Differential spectroscopy
7. Derivative spectroscopy
8. Area under curve method.

1.2.2.5 Different spectroscopic methods

The assay of an absorbing substance may be quickly carried out by preparing a solution in a transparent solvent and measuring its absorbance at a suitable wavelength. The wavelength normally selected is a wavelength of maximum absorption (λ_{max}), where small errors in setting the wavelength scale have little effects on the measured absorbance.

1.2.2.5.1 Assay of substances in single component samples

Absorption spectroscopy is one of the most useful tools available to the chemist for quantitative analysis. The most important characteristics of photometer and spectrophotometric method are high selectivity and ease of convenience.

Quantitative analysis (assay of an absorbing substance) can be done using following methods.

- Use of $A_{1\,\mathrm{cm}}^{1\%}$ values
- Use of calibration graph (multiple standard method)
- By single or double point standardization method.

1.2.2.5.1.1 Use of $A_{1\,cm}^{1\%}$ values

This method can be used for estimation of drug from formulations or raw material, when reference standard not available. The use of standard value $A_{1\,\mathrm{cm}}^{1\%}$ avoids the need to prepare a standard solution of the reference substance in order to determine its absorptivity, and is of advantage in situations where it is difficult or expensive to obtain a sample of the reference substance.

1.2.2.5.1.2 Use of calibration graph

In this procedure the absorbance of a number of standard solutions of the reference substance at concentrations encompassing the sample concentrations are measured and a calibration graph is constructed. The concentration of the analyte in the sample solution is read from the graph as the concentration corresponding to the absorbance of the solution. Calibration data are essential if the absorbance has a non-linear relationship with concentration, or if the absorbance or linearity is dependent on the assay conditions. In certain visible spectrophotometric assays of colourless substances, based upon conversion to coloured derivatives by heating the substance with one or more reagents, slight variation of assay conditions, e.g. P^H, temperature and time of heating, may rise to a significant variation of absorbance, and experimentally derived calibration data are required for each set of samples.

1.2.2.5.1.3 Single or double point standardization

The single point procedure involves the measurement of the absorbance of a sample solution and of a standard solution of the reference substance. The standard and the sample solution are prepared in similar manner; ideally the concentration of the standard solution should be close to that of the sample solution. The concentration of the substance in the sample is calculated using following formula.

$$C_{test} = A_{test} \times C_{std} / A_{std}$$

Where,

C_{test} and C_{std} are the concentration in the sample and standard solutions respectively.

A_{test} and A_{std} are the absorbance of the sample and standard solutions respectively.

In double point standardization, the concentration of one of the standard solution is greater than that of the sample while the other standard solution has a lower concentration than the sample. The concentration of the substance in the sample solution is given by

$$C_{test} = \frac{(A_{test} - A_{std1})(C_{std1} - C_{std2}) + C_{std1}(A_{std1} - A_{std2})}{A_{std1} - A_{std2}}$$

Where,

C_{std} is the concentration of the standard solution.

A_{test} and A_{std} are the absorbance of the sample and standard solution respectively.

Std_1 and Std_2 are the more concentrated standard and less concentrated standard respectively.

1.2.2.5.2 Assay of substances in Multicomponent Samples

The spectrophotometric assay of drugs rarely involves the measurement of absorbance of samples containing only one absorbing component. The pharmaceutical analyst frequently encounters the situation where the concentration of one or more substances is required in samples known to contain other absorbing substances which potentially interfere in the assay. If the recipe of the sample formulation is available to the analyst, the identity and concentration of the interferants are known and the extent of interference is the assay may be determined. Alternatively, interference which is difficult to quantify may arise in the analysis of formulations from manufacturing impurities, decomposition products and formulation excepients. Unwanted absorption from this source is termed irrelevant absorption and, if not removed, imparts a systematic error to the assay of the drug in the sample.

The basic of all the spectrophotometric techniques for multicomponent samples is the property that at all wavelengths:

a. The absorbance of a solution is the sum of absorbance of the individual components, or
b. The measured absorbance is the difference between the total absorbance of the solution in the sample cell and that of the solution in the reference (blank) cell.

The various spectroscopic techniques used for multi-component analysis are the fallowing

1. Simultaneous equation method
2. Absorption ratio method
3. Geometric correction method
4. Absorption correction method
5. Orthogonal polynomial method

6. Differential spectroscopy

7. Derivative spectroscopy

8. Area under curve method.

1.3 CHROMATOGRAPHY *(Sharma B.K., 2000; Gurdeep R. Chatwal et al., 2007)*

Chromatography may be regarded as "an analytical technique employed for the purification and separation of organic and inorganic substances". It is also found useful for the fractionation of complex mixture and separation of closely related compounds such as isomers and isolation of unstable substances. Chromatography technique is based on the difference in the rate at which the components of a mixture move through a porus medium (stationary phase) under the influence of some solvent or gas (mobile phase).

The chromatographic method of a separation in general involves the following steps:

a. Adsorption or retention of a substance or substance on the stationary phase.

b. Separation of the adsorbed substance by the mobile phase.

c. Recovery of the separated substance by a continuous flow of the mobile phase. The method being called elution.

d. Quantitative and qualitative analysis of the eluted substance.

1.3.1 Types Of Chromatography

(*http://www.buzzle.com/article/types-of-chromatography.html*)

Chromatography techniques are roughly classified on the basis of purpose for which they are used and method developed. The different types of laboratory techniques used in the separation of mixtures are grouped under an umbrella term, chromatography. The process through which constituents of a mixture are separated and analyzed by physical means is referred to as chromatography. Apart from the

different criteria of classification of chromatography discussed below, the basic criterion is the purpose for which this process is carried out. On the basis of this criterion, the process of chromatography is classified into analytical and preparative. The former is carried out for the purpose of measuring the amount of an analyte present in a mixture. On the other hand, preparative chromatography is used for separating the components of a mixture for their further use. Depending on the techniques used in chromatography, the process is broadly classified as adsorption and partition chromatography.

1.3.1.1 Adsorption chromatography

In this form of chromatography, the chemical mixtures in question are passed over an adsorbent bed. Different compounds present in the mixture get adsorbed on the bed at different rates. This process is mostly carried out for analytical separation. Adsorption chromatography is further divided into 'affinity' and 'ion-exchange' chromatography.

1.3.1.2 Ion-exchange chromatography

The mechanism of ion-exchange which is used in this form of chromatography allows carrying out the segregation of analytes. This kind of segregation/separation can be performed in 2 different modes, i.e. planar and column. Separation of charged compounds like peptides, amino acids, proteins, etc. takes place through a charged stationary phase.

1.3.1.3 Column chromatography

The column chromatography technique uses a set-up in which the stationary phase is placed in a column. There are two ways through which the stationary phase is positioned in a column: either it entirely fills the column or lines the walls of the column.

1.3.1.4 Planar chromatography

The stationary phase is placed on a plane surface. The set-up is unlike the one used in column chromatography where stationary phase is placed in a column. Here, a plane surface is used. The plane surface could be anything from paper to glass.

1.3.1.5 Affinity chromatography

The non-covalent interaction which takes place between the analyte in question and certain molecules is the basis of working of affinity chromatography. Purification of proteins bound to tags is conducted with this technique.

1.3.1.6 Partition chromatography

In this separation technique, components of the given mixture are separated through the use of partition of a solute between two solvents. In the process, one of the solvents is immobilized by means of a substance present in the filter paper or column.

1.3.1.7 Gel Filtration chromatography

This technique is also known as gel permeation or size exclusion chromatography. Molecules of the mixture in question are separated on the basis of their size. Technically speaking, the process of separation is carried out on the basis of hydrodynamic diameter (size) of molecules. Larger molecules of the mixture are unable to enter the pores of media; therefore, molecules are washed out quickly. On the other hand, smaller molecules take more time to elute because they are able to enter the pores of media.

1.3.1.8 High performance liquid chromatography

In this type of chromatography, separation of compounds is carried out on the basis of their idiosyncratic polarities. Interaction of these compounds with the stationary phase of the column too is considered. Equipment needed for carrying out

high performance liquid chromatography includes a pump (used for moving the mobile phase and analyte through the column), stationary phase and a detector. Retention time for the analyte is also provided by the detector. Depending on the strength of interactions taking place between the analyte and stationary phase, retention time can vary.

1.3.1.9 Gas chromatography

This form of chromatography uses cylinders wherein gas is stored under pressure. These gases do the work of carrying the solute. The carrier gas that is commonly used in this chromatography is helium. Flame ionization detectors and thermal conductivity are used in gas chromatography. There are three sub-types of gas chromatography which include the following: gas-liquid chromatography, gas adsorption chromatography and capillary gas chromatography. In gas-liquid chromatography, an inert porous solid is used as the stationary phase. The stationary phase used in gas chromatography is a bed formed by an adsorbent. In capillary gas chromatography, the adsorbents form a layer on fused silica or glass which line the capillary walls.

1.3.1.10 Pyrolysis gas chromatography

This method of chromatography makes use of pyrolysis i.e. decomposition of the sample with the help of thermal power. The process of pyrolysis is followed by the regular procedure of gas chromatography. Resistive heating, inductive heating and heating in isothermal furnace are the three methods used for carrying out pyrolysis in this technique. The volatile fragments formed by heating (at a temperature of 600-1000 °C) are separated by means of gas chromatography.

1.3.1.11 Reverse-phase chromatography

This technique employs a method which is just opposite to that of normal phase chromatography. In reverse-phase chromatography, the stationary phase is made up of hydrophobic compounds; they attract the hydrophobic compounds present in the mobile phase. Here, the polarity of mobile phase is reduced in order to allow the hydrophobic molecule to elute. The technique of chromatography which is meant for separation of compounds from mixtures thus, holds immense importance in fields like biochemistry, biotechnology and many others. An attempt to list as many types of chromatography as possible is made in this write-up.

1.3.2 High Performance Liquid Chromatography *(Sharma B.K., 2006)*

The high performance liquid chromatography is thus a method of separation in which the stationary phase is contained in a column, one end which is attached to a source of pressurized liquid eluent (mobile phase).

The choice of mobile phase is very important in HPLC and the eluting power of the mobile phase is determined by it's over all polarity of the stationary phase and the nature of sample components. For normal phase separations the eluting power increases with increasing polarity of the solvent, but for reversed phase separations, eluting power decreases with increasing solvent polarity.

1.3.2.1 HPLC separation modes

(http://www.justchromatography.com/chromatography/hplc-separation-modes)

Chromatographic Mode	Abrv.	Column Type	Mobile Phase	Application
Reverse-phase chromatography	RPC	Non-polar (e.g. C_{18})	Polar mixture of water and organic solvent (e.g.	Water-soluble samples

Chromatographic Mode	Abrv.	Column Type	Mobile Phase	Application
			acetonitrile)	
Normal-phase chromatography	NPC	Polar (e.g. unbonded silica)	Less-polar (than stationary phase) mixture of organic solvents (e.g. hexane, ethyl ether, chloroform, methylene chloride)	Water-insoluble samples, isomer separation, and preparative HPLC
Non-aqueous reverse-phase chromatography	NARP	Non-polar (e.g. C_{18})	Mixture of organic solvents (e.g. ACN + methylene chloride)	Very hydrophobic samples
Hydrophilic interaction chromatography	HILIC	Polar (e.g. silica or amide-bonded)	Mixture of water organic solvents (e.g. ACN + H_2O	Highly polar samples that are poorly retained by reverse-phase mode
Ion-exchange chromatography	IEC	Usually an organic resin that has charged groups able to bind ions of opposite charge	Aqueous solution of a salt with buffer.	Separating ionizable samples, large biomolecules (e.g. proteins, carbohydrates)
Ion-pair chromatography	IPC	Non-polar (e.g. C_{18})	Polar mixture of water and organic solvent (e.g. ACN) w/ an ion-pairing reagent (e.g. alkylsulfonates, trifluoroacetic acid) that interacts w/ sample ions of opositecharge	Acids and bases that are weekly retained by reverse-phase
Size-exclusion chromatography	SEC	Inert	Aqueous or organic	Large biomolecules, polymers. Separates by

Chromatographic Mode	Abrv.	Column Type	Mobile Phase	Application
				molecular weight.

1.3.2.2 Elution techniques (*Sharma B.K., 2006)*

Two types of elution techniques generally used. They are,

i) Isocratic elution

One particle solvent or mixture is pumped through the whole analysis.

ii) Gradient elution

For some determinations the solvent composition may be altered gradually gradient elution system can be classified as low pressure and high pressure system. In low pressure gradient elution system the eluent components are miner proportion varying with time at low pressure and the mixture is pumped in order to be delivered at high pressure to the column. In high pressure gradient elution system components or mixtures of fixed composition are each pumped by separate pump and then mixed at high pressure in a ratio varying with time.

1.3.2.3 HPLC stationary phases *(Robert D. Braun, 1986)*

HPLC can be performed by using the stationary phases. Generally the stationary phases are packed into a stainless steel column of 10-, 15-, 25-, 50-, 100-cm length with a diameter which is usually between 2 and 6 mm for analytical columns. Alumina is solid adsorbent which is widely used as a column packing material because alumina is basic it retains acidic compounds. Silica gel is also used as a packing material in the form of pure particles and as a pellicle on a solid support. A

pellicle is a thin layer or coating on a surface generally the pellicles used for HPLC have thickness about 1µm on glass beds which have diameter about 40mm.

Mobile phases

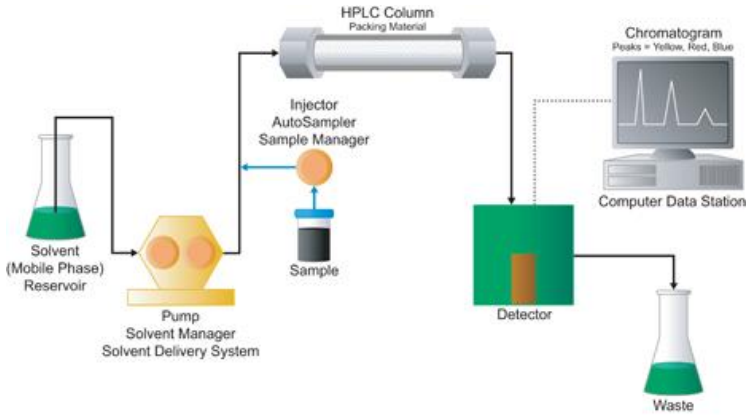
In HPLC, for solid adsorbent and liquid stationary phases (both normal and reverse phase) mixed solvents are used as the mobile phase .Generally the mobile phase consists of a mixture of polar solvents such as alcohol and non-polar solvents such as hydro carbons.

The mobile phase must be chosen so as not to interfere with the measurement by the detector. For example, if an UV absorption detector is used the solvent cannot absorb UV radiation. Mixtures of methanol, ethanol or propanol with heptane and of chloroform with heptane are popular choices of HPLC mobile phase.

1.3.2.4 Instrumentation*(Willard et al., 1986)*

The general instrumentation for HPLC incorporates the following components:

i. Mobile phase reservoir pump
ii. Injector
iii. Column
iv. Detector and Data system



Schematic diagram of HPLC*(www.chromotech.com/ab/in/tech)*

1.3.2.4.1 Mobile phase reservoir pump

The mobile phase must be delivered to the column over a wide range of flow rates and pressures to permit the use a wide variety of organic and inorganic solvents. The pump it seals and all connections must be made of materials chemically resistant to the mobile phase. A degasser is needed to remove the dissolved air and other gases from the solvent. Another desirable feature is the solvent delivery system is the capability for generating a solvent gradient.

In HPLC pump should be able to operate to at least 100atm (1500 psi). A pressure suited to less expensive chromatographs however, 400atm (6000psi) is a more desirable pressure limit.

Standard HPLC pump requirements are,

a. Flow rate range 0.01 to 100 ml/ min.
b. Pressure range: 1 to 5000 psi.
c. Flow rate stability not more than 1%
d. Pressure pulsations less than 1%

The types of pumps used are,

1. Reciprocating Piston Pumps
2. Syringe-Type Pumps
3. Constant-Pressure Pumps

The common problems in pumping liquids are,

a. Solvent degassing
b. Corrosion
c. Compressibility

1.3.2.4.2 Injectors

In liquid chromatography, liquid samples may be injected directly and solid samples need only be dissolved in an appropriate solvent. The solvent need not be the mobile phase, but frequently it is judiciously chosen to avoid detector interference, column/component interference and loss in efficiency or all of these. It is always best to remove particles from the sample by filtering or centrifuging since continuous injections of particulate material will eventually cause blockage of injection devices or columns. Standard HPLC injector should be accurate in injecting volume in the range of 0.1 to 100 ml with high reproducibility and under high pressure (up to the 4000 psi) and should produce minimum band broadening and should minimize possible flow disturbances.

1.3.2.4.3 HPLC Columns

HPLC columns are packed with very fine particles (usually a few microns in diameter). The very fine particles are required to attain the low dispersion that give the high plate counts expected of modern HPLC. Plate counts in excess of 25,000 plates per column are possible with modern columns because of the dispersion associated with injection valves, detectors, data acquisition systems and the dispersion due to the higher molecular weight of real samples as opposed to the common test samples. LC columns, achieve their separation by exploiting the different intermolecular forces between the solute and the stationary phase and those between the solute and the mobile phase. The column will retain those substances that interact more strongly with the stationary phase than those that interact more strongly with the mobile phase. The main consideration with HPLC is the much wider variety of solvents and packing materials that can be utilized because of the much lower quantities of both which are required. In particular, very expensive optically pure

compounds can be used to make chiral HPLC stationary phases and may even be used as (disposable) HPLC solvents.

Different types of columns are used. They are,

1. Analytical column
2. Short column
3. Narrow bore column
4. Guard column
5. Inline filters

Analytical column variables are as follows:

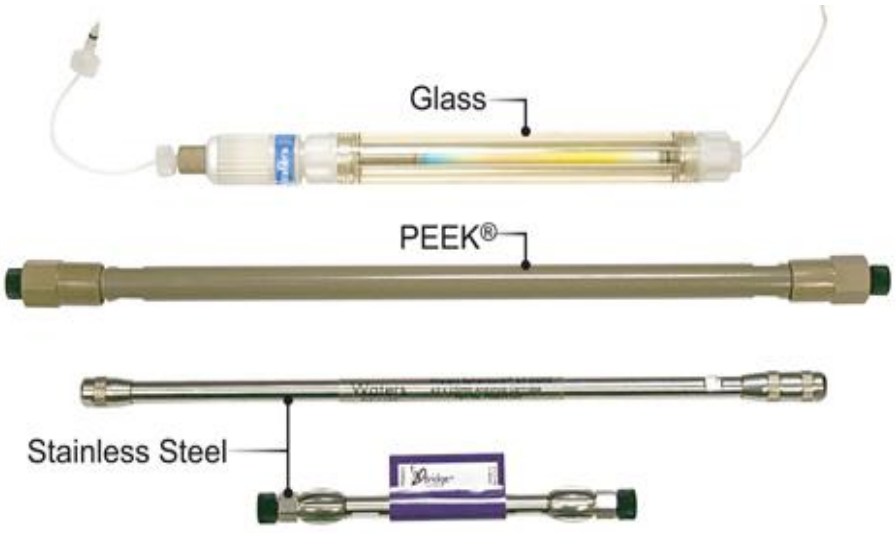
a. Length (10-30 cm)

b. ID (4-10 mm)

c. Packing (many kinds)

d. Particles sizes (3-10 µm)

e. Most common columns 250mmx4.6mm i.e. with 5µm particle size.



(http://www.waters.com/webassets/cms/category/media/other_images/primer_M-_Columnexamples.jpg)

1.3.2.4.4 Detectors

Optical detectors are most frequently used. Current LC detectors have wide dynamic range normally allowing both analytical and preparative scale runs on the same instrument.

Basic detector requirements

An ideal LC detector should have the following properties:

1. Low drift and noise level (particularly crucial in trace analysis).
2. High sensitivity.
3. Fast response.
4. Wide linear dynamic range (this simplifies quantification).
5. Low dead volume (minimal peak broadening).
6. Cell design which eliminates remixing of the separated bands.
7. Insensitivity to changes in type of solvent, flow rate, and temperature.
8. Operational simplicity and reliability.
9. It should be tunable so that detection can be optimized for different compounds.

On-line detectors

A. Refractive index.
B. UV/Visible Fixed wavelength.
C. UV/Visible Variable wavelength.
D. UV/Visible Diode array.
E. Fluorescence.
F. Conductivity.
G. Mass-spectrometric (LC/MS).

Off-line detector

A. FTIR spiral disk monitor.

Noise and drift

The problem is to distinguish between the actual component and artifact caused by the pressure fluctuation, bubble, compositional fluctuation, etc. If the peaks are fairly large, one has no problem in distinguishing them. However, the smaller the peak, the more important is that the baseline be smooth, free of noise and drift. Baseline noise is the short time variation of the baseline from a straight line. Noise is normally measured "peak-to-peak": i.e., the distance from the top of one such small peak to the bottom of the next. Noise is the factor which limits detector sensitivity. In trace analysis, the operator must be able to distinguish between noise spikes and component peaks. The baseline should deviate as little as possible from a horizontal line. It is usually measured for a specified time, e.g., 1/2 hour or one hour and called drift. Drift usually associated to the detector heat-up in the first hour after power-on.

Data systems

The main goal in using electronic data systems is to increase analysis accuracy and precision while reducing operator attention. In routine analysis, where no automation is needed, a pre-programmed computing integrator may be sufficient. For higher control levels, a more intelligent device is necessary, such as a data station or minicomputer.

The advantages of intelligent processors in chromatographs are,

- Additional automation options become easier to implement.
- Complex data analysis becomes more feasible.
- Software safeguards can be designed to reduce accidental misuse of the system.

1.3.2.6 Summary of HPLC operation (*Dong., 2006)*

1. Filter and degas mobile phase.
2. Prime pump, rinse column with strong solvents and equilibrate column.
3. Purge injection and make sure there are no air bubbles in the sample syringe.
4. Perform system suitability test.
5. Analyze sample.
6. Process and report data.
7. Rinse column and shut down pump and lamps.

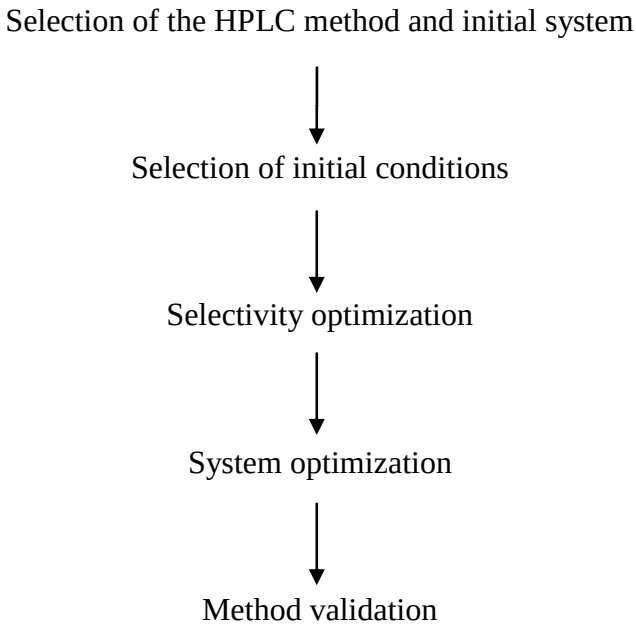
1.3.2.7 Checking for problems *(Lloyd R.Snyderet al., 1997)*

As method development proceeds, various problems can arise, some of which are listed below.

Problems	**Comments**
Low plate numbers	Poor choice of column, poor peak shape effect.
Column variability	Poor choice of column, secondary retention effect.
Short column life	Poor choice of column, need for sample pretreatment.
Retention drift	Insufficient column equilibration, need for sample pre-treatment, loss of bonded phase.
Poor quantitative precision	Need for better calibration.

1.3.2.8 HPLC method development *(LloydR.Snyder et al., 1997)*

The wide variety of equipment, columns, eluent and operational parameters involved makes high performance liquid chromatography (HPLC) method development seem complex. The process is influenced by the nature of the analytes. The different stages in method development was given in the following flow chart



Quantitative analysis

The four primary techniques for quantitation are

a. Normalized peak area

b. External standard calibration method

c. Internal standard calibration method

d. method of standard addition

a.Normalized peak area

After completion of a run and the integration of all significant peaks in the chromatogram, the total peak area can then be calculated. The area percent of any individual peak is referred to as the normalized peak area. The technique of normalized peak area is actually not a calibration method per se, since there is no comparison to a known amount for any peak in the chromatogram. However, this technique is widely used to estimate the relative amounts of small impurities or degradation compounds in a purified material.

b. External standard calibration method

The most general method for determining the concentration of an unknown sample is to construct a calibration plot using external standards. Standards solutions are prepared at known concentrations. A fixed volume of each standard solution is injected and analyzed, and the peak responses are plotted Vs concentration. The standard solutions are referred to as external standards, since they are prepared and analyzed in separate chromatograms from those of the unknown samples. Unknown samples are then prepared, injected and analyzed exactly in the same manner.

c. Internal standard calibration method

Another technique for calibration involves the addition of an internal standard to the calibration solutions and samples. The internal standard is a different compound form the analyte, but one that is well resolved in the separation. The internal standard can compensate for changes in sample size or concentration to instrumental variations. With the internal standard method, a calibration plot is produced by preparing and analyzing calibration solutions containing different concentration of the compound of interest with a fixed concentration of the internal added.

The Internal standard comply the following requirements:

i. Well resolved from the compound of interest and other peaks.
ii. Similar retention (k) to the analyte.
iii. Should not be in the original sample.
iv. Should mimic the analyte in any sample preparation steps.
v. Does not have to be chemically similar to analyte.
vi. Commercially available in high purity.
vii. Stable and unreactive with sample or mobile phase.

viii. Should have similar detector response to the analyte for the concentration used.

d. Method of standard addition

A calibration standard ideally should be prepared in a blank matrix of drug formulation components without the drug substance or an animal without added compound usually can be used for standard calibration solutions. The method of standard addition is most often used in trace analysis. In this approach, different weights of analyte(s) are added to the sample matrix, which initially contains an unknown concentration of the analyte. Extrapolation of a plot of response found for the standard addition calibration concentration to zero concentration defines the original concentration in the un spiked sample.

1.3.2.9 System Suitability Parameters
(Lloyd R.Snyder, 1997; Beckette and Stenlake, 2007)

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated.

The parameters that are affected by the changes in chromatographic conditions are,

- Column capacity factor *(K^1)*
- Resolution (R_s)
- Selectivity (α)
- Column efficiency (N) and
- Peak asymmetry factor (A_s)
- Tailing factor (T)

Column capacity factor *(K^1)*

The retention of a drug with a given packing material and eluent can be expressed as retention time or retention volume, but both of these are dependent on flow rate, column length and column diameter. The retention is best described as a column capacity ratio (K), which is independent of these factors. The column capacity ratio of a compound (A) is defined as

$$K_A = \frac{V_A - V_0}{V_0} = \frac{t_A - t_0}{t_0}$$

Where,

V_A = Elution volume of A

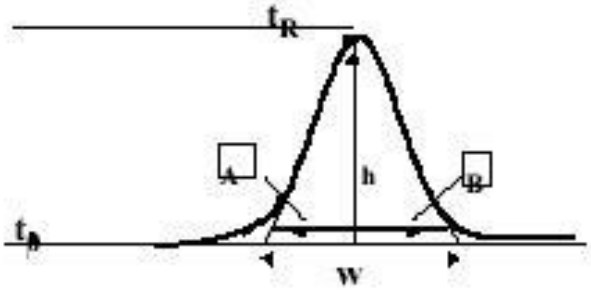
V_0 = Elution volume of a non-retained compound (void volume)

At constant flow rate, retention times (t_A and t_o) can be used instead of retention volumes. Retention data is sometimes expressed, relative to a known internal standard (B). The ratio of retention times (t_A/t_B) can be used, but the ratio of adjusted retention times $\left(\frac{t_A - t_o}{t_B - t_o}\right)$ is better when data need to be transferred between different chromatographs.

The values of 'k' of individual bands are increase or decrease with changes in solvent strength. In reversed phase HPLC, solvent strength increases with the increase in the volume of organic phase in the water / organic mobile phase. Typically an increase in percentage of the organic phase by 10 % by volume will decrease k' of the bands by a factor of 2-3.

RETENTION FACTOR or CAPACITY RATIO

$$k' = \frac{t_R - t_0}{t_0} \qquad k' = \phi \frac{C_s}{C_m}$$



Resolution (R_s)

The resolution, R_s of two neighbouring peaks is defined by the ratio of the distance between the two peak maxima. It is the difference between the retention times of two solutes divided by their average peak width. For baseline separation, the ideal value of R_s is 2.0. It is calculated by using the formula,

$$R_f = \frac{Rt_2 - Rt_1}{0.5\,(W_1 + W_2)}$$

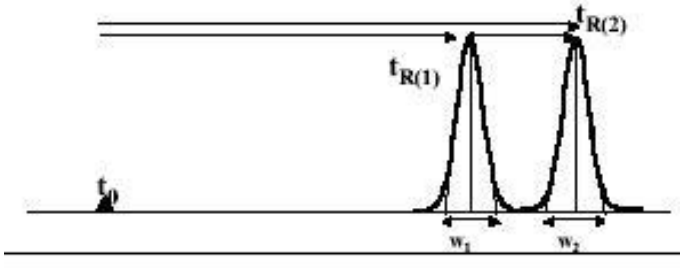
Where,

Rt_1 and Rt_2 are the retention times of components 1 and 2

W_1 and W_2 are peak widths of components 1 and 2.

EXPERIMENTAL RESOLUTION

$$Rs = \frac{t_{R(2)} - t_{R(1)}}{1/2\,(W_1 + W_2)}$$

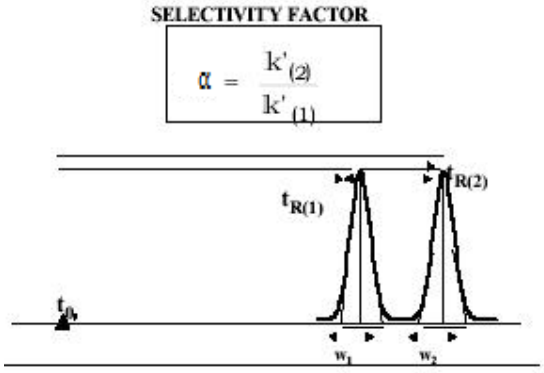


Selectivity (α)

The selectivity (or separation factor), α, is a measure of relative retention of two components in a mixture. The ideal value of selectivity is 2. It can be calculated by using the formula,

$$\alpha = \frac{V_2 - V_0}{V_1 - V_0}$$

Where, V_0 is the void volume of the column and V_2 and V_1 are the retention volumes of the second and the first peak, respectively.



Column efficiency

Efficiency, N, of a column is measured by the number of theoretical plates per meter. It is a measure of band spreading of a peak. Smaller the band spread, higher is the number of theoretical plates, indicating good column and system performance. Columns with N ranging from 5,000 to 100,000 plates/meter are ideal for a good system. Efficiency is calculated by using the formula,

$$N = 16\frac{Rt^2}{W^2}$$

Where,

Rt is the retention time and W is the peak width.

Peak asymmetry factor (A_s)

Peak asymmetry factor, $A_s$canbe used as a criterion of column performance. The peak half width b of a peak at 10 % of the peak height, divided by the corresponding front half width a gives the asymmetry factor.

$$A_S = \frac{b}{a}$$

Tailing factor (T)

The tailing factor T, a measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increases as tailing becomes more pronounced.

In some cases, values less than unity may be observed. As peak asymmetry increases,integration, and hence precision becomes less reliable.

$$\mathrm{T} = \frac{\mathrm{W}_{0.09}}{2\mathrm{f}}$$

Where,

$W_{0.05}$ = width of peak at 5% height

f = Distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline.

Limit ≤ 2 is preferable.

Height Equivalent to a Theoretical Plate (HETP)

A theoretical plate can be of any height, which decides the efficiency of separation. If HETP is less the column is more efficient. If HETP is more, the column is less efficient. The height equivalent to a theoretical plate (HETP) is given by

$$\mathrm{HETP} = \frac{\text{Length of the column}}{\text{No. of the theoretical plates}}$$

1. VALIDATION *(Michael E. Swartz et al., 2009)*

The validation is divided into different types. They are,

Prospective Validation

This method is employed when historical data of the product is not available or is not sufficient and in process and finished product testing is not adequate to ensure reproducibility or high degree of compliance to product likely attributes.

Retrospective Validation

This provides trend of comparative result (i.e.) review and evaluation of existing information for comparison when historical data is sufficient and readily available.

Concurrent Validation

Based on information generated during implementation of a system for this extensive testing and monitoring are performed as part of initial run of the method.

Re-Validation

Revalidation provides the evidence that changes in a process and are the process environment, introduced either intentionally or unintentionally, do not adversely affect process characteristic and product quality.

There are two basic categories of revalidation. Revalidation in case of known change (including transfer of process from one company to another or from one site to another). Periodic revalidation carried out at scheduled intervals.

Reasons/Purpose of Validation

- Setting standards of evaluation procedures.
- Taking appropriate action in case of non-compliance.
- Retrospective validation is useful for trend comparison of results compliance to cGMP/cGLP.

- Closer interaction with pharmacopoeia forum to address analytical problems. Enables scientist to communicate scientifically and effectively on technical matters.

1.4.1 Analytical Parameters Used In Assay Validation As Per ICH Guidelines

(Code Q2A; Q2B, ICH Guidelines 1994 and 1996)

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. A tabular summation of the characteristics applicable to identification, control of impurities and assay procedures is included.

Types of Analytical Procedures to be validated

The discussion of the validation of analytical procedures is directed to the four most common types of analytical procedures

Identification Tests

- ❖ Quantitative tests for impurities content.
- ❖ Limit tests for the control of impurities.
- ❖ Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product.

A brief description of the types of tests considered in this document is provided below.

Identification tests are intended to ensure the identity of an analyte in a sample this is normally achieved by comparison of a property of the sample (example spectrum, chromatographic behavior, chemical reactivity etc.) to that of a reference standard.

Testing for impurities can be either a quantitative test or a limit test for the impurity in a sample. Either test is intended to accurately reflect the purity

characteristics of the sample. Different validation characteristics are required for a quantitative test than for a limit test.

Assay procedures are intended to measure the analyte present in a given sample. In the context of this document, the assay represents a quantitative measurement of the major component(s) in the drug substance. For the drug product, similar validation characteristics also apply when assaying for the active or other selected component(s). The same validation characteristics may also apply to assays associated with other analytical procedures.

The objective of the analytical procedure should be clearly understood since this will govern the validation characteristics which need to be evaluated.

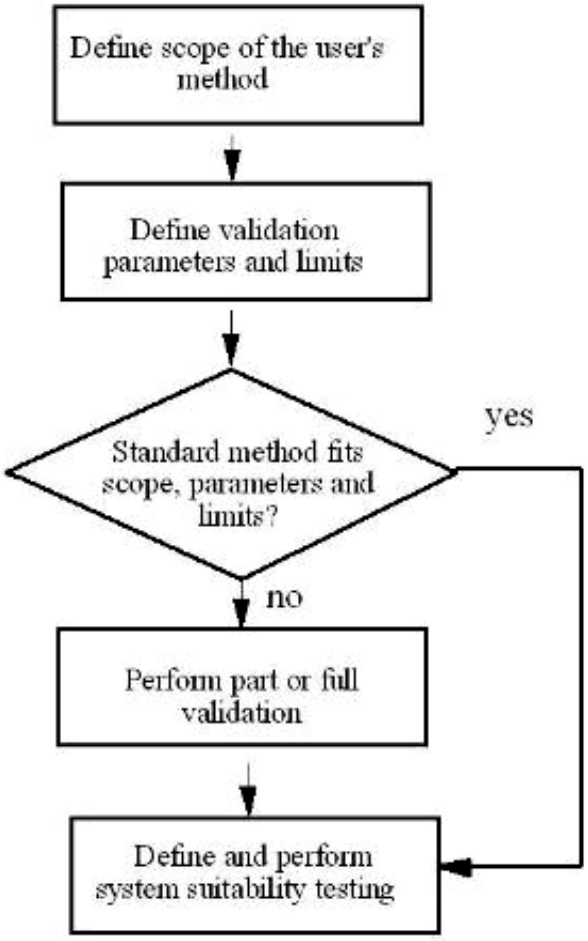


Figure: **Workflow for Evaluation and Validation of Standard Methods**

Typical Validation Parameters

- Accuracy
- Precision
- Range
- Specificity
- Linearity
- Detection Limit
- Quantification Limit
- Ruggedness
- Robustness

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or on an accepted reference value and the value found.

Precision

It expresses as degree of agreement among individual test results when procedure or method is applied to a homogeneous sample, usually expressed as SD or RSD. It is a measure of degree of repeatability or reproducibility under normal conditions. A more comprehensive definition proposed by the ICH divides precision into three types

1. Repeatability.
2. Intermediate Precision.
3. Reproducibility.

Range

The range of a method can be defined as the upper and lower concentrations for which the analytical method has adequate accuracy, precision and linearity. The range of concentrations examined will depend on the type of method and its use.

Specificity

Ability of the method to measure accurately and specifically the analyte of interest in presence of matrix and other components likely to be present in the sample matrix and impurities, degradation products and other related substances. For this, one may compare the test results of analysis of samples containing other ingredients or impurities or degradation products or related substances or placebo ingredients with those obtained from analysis of sample without these, i.e., the method must allow distinct analytical measurement of analyte of interest and exclusion of all other relevant interferences. If the impurities or degradation products or potential contaminants are not available one can apply a proposed method to the strain and stress (heat, light, humidity) samples. Degree of agreement among results will explain specificity of the method. If the impurities or degradation products are not available, one may carryout additional purity tests by chromatography-HPLC or HPTLC.

Linearity

The linearity of an analytical procedure is its ability to obtain test results, which are directly proportional to the concentration of analyte in the sample. Linearity can be assessed by performing single measurements at several analyteconcentrations,A linearity correlation coefficient above 0.999 is acceptable for most methods, especially for major components in assay methods. The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample.

Detection limit

The Detection Limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantities as an exact value. The detection limit (LOD) may be expressed as

$$\mathrm{LOD} = \frac{3.3\sigma}{S}$$

Where,

σ = the standard deviation of the response.

S = the slope of the calibration curve (of the analyte).

Quantification limit

LOQ is defining as the lowest concentration of the substance (analyte) in a sample that can be estimated quantitatively with acceptable precision, accuracy and reliability by a given method under stated experimental conditions. Quantification Limit (LOQ) may be expressed as

$$\mathrm{LOQ} = \frac{10\sigma}{S}$$

Where,

σ = the standard deviation of the response.

S = the slope of the calibration curve (of the analyte).

Ruggedness

It is the measure of the capacity of the analytical method to remain unaffected by small but deliberate variations in procedure. It provides an indication about variability of the method during normal laboratory conditions.

Robustness

The concept of robustness of an analytical procedure has been defined by the ICH as "A measure of its capacity to remasin unaffected by small, but deliberate variations in method parameters". The most important aspect of robustness is to develop methods that allow for expected variations in the separation parameters.

General acceptance limits

S.No.	**Characteristics**	**Acceptance Criteria**
1	Accuracy	Assay limit- 98-102% Recovery - 80,100,120%.with a deviation of $\pm$ 2
2	Precision A)Repeatability b)Intermediate precision	 % RSD < 2 % RSD < 2
3	Specificity/ selectivity	No interference
4	Detection Limit	S/N > 2 or 3
5	Quantitation Limit	S/N > 10
6	Linearity	r > 0.999
7	Range	80-120%
8	Stability	>24hr or > 12hr

1.5 BASIC STATISTICAL PARAMETERS

(*Gupta, et al., 1995; Bolton, et al., 2004; Mendham, et al., 1994*)

Statistical techniques have been widely used in many diverse areas of scientific investigation. Statistical applications have been recognized as crucial to quality control procedure, test, specification and definitions. Principle of modern analytical techniques and skill in their application are necessary attribute of the successful pharmaceutical analyst, thus does not ensure the satisfactory solution of all the problem that may encountered. Some auxiliary knowledge methods those can aid the analyst in designing experiment, collecting data, and interpreting the result.

1.5.1 Linear Regression

Linear regression is a statistical technique that defines the functional relationship between two variables by best-fitting straight line. Once a linear relationship has been shown to have a high probability by the value of the correlation coefficient 'r', then the best straight line through the data points has to be estimated. This can often be done be done by visual inspection of the calibration graph, but in many cases it is far more sensible to evaluate the best straight line by linear regression (the method of least squares).

The equation of straight line is

$$y = mx + c$$

Where, y the dependent variable is plotted as result of changing x, the independent variable.

To obtain the regression line 'y on x' the slope 'm' of the line and the intercept 'c' on the y axis are given by the following equation.

$$m = \frac{N\sum xy - (\sum x)(\sum y)}{N\sum x^2 - (\sum x)^2} \text{ and } \qquad c = \frac{(\sum y)(\sum x^2) - (\sum x)(\sum xy)}{N\sum x^2 - (\sum x)^2}$$

1.5.2 Correlation Coefficient (r)

It is a procedure commonly used to characterize quantitatively the relationship between variable. Correlation is related to linear regression. To establish whether there is a linear relationship between two variables x_1 and y_1, use Pearson's correlation coefficient r.

$$r = \frac{n\sum x_1 y_1 - \sum x_1 y_1}{\{[n\sum {x_1}^2 - (\sum x_1)^2][n\sum {y_1}^2 - (\sum y_1)^2]\}^{1/2}}$$

Where n is the number of data points.

The value of r must lie between +1 and -1, the nearer it is to +1, the greater the probability that a definite linear relationship exists between the variables x and y, values close to +1 indicate positive correlation and values close to -1 indicate negative correlation values of 'r' that tend towards zero indicate that x and y are not linearly related (they made be related in a non-linear fashion).

1.6.3 Standard Deviation (SD)

It is commonly used in statistics as a measure of precision statistics as a measure of precision and is more meaningful than is the average deviation. It may be thought of as a root-mean-square deviation of values from their average and is expressed mathematically as

$$S = \sqrt{\frac{\sum_{i=1}^{i=n}(x_i - \overline{x})}{N-1}}$$

Where,

S is standard deviation.

ZIf N is large (50 or more) then of course it is immaterial whether the term in the denomination is N -1 or N

Σ = sum

$\bar{x}$ = Mean or arithmetic average.

$x - \bar{x}$ = deviation of a value from the mean.

N = Number of observations.

1.5.4 Percentage Relative Standard Deviation (%RSD)

It is also known as coefficient of variation (CV). It is defined as the standard deviation (SD) expressed as the percentage of mean.

$$CV \text{ or } \% \text{ RSD} = \frac{S.D}{\bar{x}} \times 100$$

Where,

SD = the standard deviation,

$\bar{x}$ = Mean or arithmetic average.

The variance is defined as S^2 and is more important in statistics than S itself. However, the latter is much more commonly used with chemical data.

1.5.5 Standard Error of Mean (SE)

Standard error of mean can be defined as the value obtained by division of standard deviation by square root of number of observations. It is mathematically expressed as

$$S.E. = \frac{S.D.}{\sqrt{n}}$$

Where,

SD = Standard deviation.

n = number of observation

1.6.6 Confidence Interval (CI)

A confidence interval gives an estimated range of values which is likely to include a unknown population parameter, the estimated range being calculated from a

given set of sample data. A confidence interval with a particular confidence level (95% selected by the user) is intended to give the assurance that, if the statistical model is correct then the interval could deliver the true value.

Confidence interval for a normal population,

$$\bar{Y} \pm \frac{Z_{\alpha/2}\ \sigma}{\sqrt{N}}$$

Where

$\bar{Y}$ = Sample mean

$Z_{\alpha/2}$ = upper $\alpha/2$ critical value of standard normal distribution

N = Size of sample

σ = Standard deviation

LITERATURE REVIEW

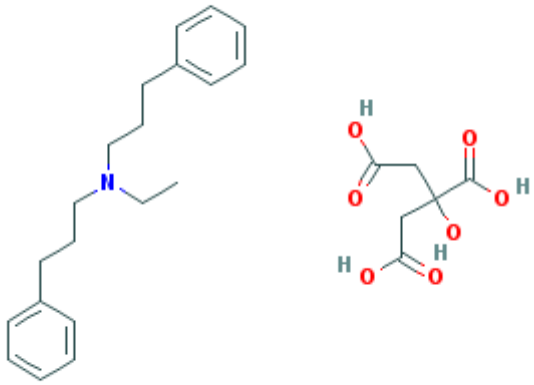
2. LITERATURE REVIEW

2.1 DRUG PROFILE

2.1.1 Alverine Citrate

(Martindale The extra Pharmacopoeia, 2005;Martindale, The complete drug reference,2005;TheMerck Index,2006; Clark's Isolation of Drugs and Poisons, 2004; db.yaozh.com/foreign/BP2009/1-2/1-2_67.pdf5/6/14; http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=21718)

Molecular structure



Chemical name

N-Ethyl-3,3-diphenyl diproyllamine citrate

Molecular formula

$C_{20}H_{27}NC_6H_8O_7$

Molecular weight

473.53

Category

It is used as an Anti- spasmodic. It is used mainly for the treatment of gastro-intestinal and biliary tract disorders, including irritable bowel syndrome.

Description

It is a white to off-white powder having a sweet odour and slightly bitter taste.

Solubility

It slightly soluble in chloroform, sparingly soluble in ethanol and very slightly soluble in ether

pka value

3.3 to 4.5

Storage

It should be stored in a well-closed container and protected from light.

Identification

1. Melting point

Standard value	Observed average value*
100 to 103	102.83

*Average of six observations

2. Infra-red spectrum was recorded and shown in figure – 1.

Mechanism of action

Agents that inhibit the actions of the parasympathetic nervous system. The major group of drugs of drug used therapeutically for this purpose is the muscarinic antagonists.

Pharmacokinetics

Alverine Citrate is observed from gastrointestinal tract following oral administration and is rapidly metabolized to active metabolite, peak plasma concentration of which occur 1 to 1.5 hours after an oral dose. Further metabolism into inactive metabolites occurs; metabolites are excreted in urine by active secretion.

Uses and administration

Alverine Citrate is an anti spasmodic that acts directly on intestinal and uterine smooth muscle. It is used for relief of smooth muscle spasm in treatment of gastro intestinal disorders such as irritable bowel syndrome. It also used in treatment of dysmenorrhoea. Alverine Citrate is given by mouth as citrate in doses of 60 mg to 120 mg one to three times daily. It has also been given by suppository as the base in dose of 80 mg two or three times daily. Alverine Citrate 67.3 mg is approximately equivalent to 40 mg of Alverine Citrate.

Adverse Effects and Precautions

Nausea, headache, pruritus, rash and dizziness have been reported. Allergic reaction, including anaphylaxis, have also occurred. Alverine Citrate is contra-indicated in patients with intestinal obstruction or paralytic ileus.

2.2 REPORTED METHODS

2.2.1. Analytical Methods

2.2.1.1. Kumar M. et al (2013) reported" **Forced degradation study of Alverine Citrate in acid by UV spectrophotometry method."**Alverine Citrate has been studied under acid hydrolytic stress condition. The study can give the results, whether the Alverine Citrate shows the stability or shows the degradation under the acidic condition. The degradation behaviour of Alverine Citrate was carried out as per the standard procedures and guidelines. The primary aim is to evaluate the stability of drug substance and formulation. Various stress condition have been used to detect the stability of Alverine Citrate. Forced acid hydrolytic degradation of Alverine Citrate was performed in bulk and solid oral formulation using O.1 M Hydrochloric acid at room temperature in different time interval such as 30 minutes, 60 minutes and

90 minutes. The resulting solutions were analyzed for content by UV spectrophotometry at maximum absorption of 257nm.

2.2.1.2. Oval N. et al (2013)) reported "**IR quantification of Alverine Citrate in bulk and oral dosage form"** Alverine Citrate is chemically N-Ethyl -3-phenyl –N-(3- phenyl propyl) propan -1-amine, commonly used as a smooth muscle relaxant, antispasmodic in irritable bowel syndrome and dysmenorrheal agent. A simple and sensitive infrared spectrophotometric method has been developed for the estimation of Alverine Citrate in capsule dosage form. The method involves the determination of Alverine Citrate by absorbance and peak area method of IR spectroscopy . The sample was analysed by KBr pellet method; IR band at 2052 Cm-1 was considered for quantification. The calibration graph were plotted with a) absorbance against concentration b) area calculated by in built software against concentration.

2.2.1.3. Niraimathi V. et al (2013) reported "**Quantification of Alverine citrate in pharmaceutical formulation by UV spectrophotometr**y". Alverine is chemically N-Ethyl-3-phenyl-N-(3-phenylpropyl)propan-1-amine,commonly used as a smooth muscle relaxant, antispasmodic in irritable bowel syndrome and dysmenorrhoea. Three new, simple, sensitive and reproducible spectrophotometric methods have been developed for the estimation of Alverine Citrate in oral dosage form. Method A and Method B involves the determination of Alverine Citrate by first derivative and second derivative spectrophotometry. The fundamental spectrum obtained was derivatized to first order and the amplitude of the negative peak was measured at 210 nm. A calibration graph was obtained by plotting concentration versus amplitude. Method C involved the determination of Alverine Citrate by area under the curve. The area under the curve between 200-225 nm was measured by

using inbuilt software. The inbuilt software calculates the area bound by the curve and the horizontal axis. The horizontal axis is selected by entering the wavelength range over which the area has to be calculated. A linearity graph was constructed by plotting concentration versus area. The sample concentration for all the methods were obtained by interpolation. The results of the analysis was validated statistically and by recovery studies. It was also found that the excipients of the formulation did not interfere in the analysis.

2.2.1.4. Rhul C. Gavhane et al (2011) reported **"Liquid chromatography/ Tandem mass spectrometry simultaneous determination of Alverine and its metabolite, monohydroxy Alveine,in human plasma:application to a pharmokinetic study".** A rapid and sensitive LC-MS-MS method for the determination of Alverine and its major metabolite, monohydroxy Alverine in human plasma using imipramine as an internal standard was developed and validated. The analytes were extracted from 0.5 ml aliquots of human plasma by solid phase extraction, using oasis cartridge. Chromatographic separation was carried on Thermo Gold C_{18} column (50 × 4.6 mm, 5 μ) at 30 °C, with isocratic mobile phase, a flow rate of 0.4 ml/ min and a total run time of 3.5 min. Detection and quantification were performed using a mass spectrometer in the selected reaction-monitoring mode with positive electro spray ionization.

2.2.1.5. Yedhupati Kondappa Naidu et al (2010) reporte "**Simultaneous Quantification of Alverine and Its Metabolite P-Hydroxy Alverine In Human Plasma with Robotic Liquid-Liquid Extraction by using Fully Validated LC-MS/MS and its Application to A Bioequivalence Study".** A sensitive and selective method is described for the quantification of Alverine and P-hydroxy Alverine inhuman plasma using ticlopidine as internal standard. The bioanalytical approach

consists of Robotic Liquid -Liquid extraction with diethyl ether: dichloromethane (70:30 chromatographic separation of 10 µl of injected sample with Thermo BDS Hypersil (C_{18}, 4.6 X 50 mm) column using acetonitrile: 10 mM ammonium formate buffer (90:10 v/v) as mobile phase followed by quantification with mass detector in selective reaction monitoring mode using electro spray ionization mode (ESI) as an interface.

2.2.1.6. Gosh C. et al (20l0) reported **"A rapid and most sensitive liquid chromatography/Tandem mass spectrometry method for simultaneous determination of Alverine and its major metabolite, para hydroxy Alverine, in human plasma: application to a pharmacokinetic and bioequivalence study"**. A rapid and highly sensitive method for the determination of Alverine) and its metabolite, Para hydroxy Alverine, in human plasma using LC-MS/MS in positive ion electro spray ionization in multiple reactions monitoring mode was developed and validated. The procedure involves a simple solid phase extraction Chromatographic separation was carried out on a Hypersil gold C_{18} column (50 mm x 4.6 mm, 5 micron) with an isocratic mobile phase and a total run time of 1.5 min.

2.2.1.7. Novel A. Gomesh et al (2009) reported "**Validated LC-MS/MS method for Determination of Alverine and one of its hydroxy metabolites in human plasma along with its application to a bioequivalence study".** The present research work involves a first of its kind rapid and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method developed and validated for simultaneous analysis of Alverine and one of its hydroxy metabolites, Para Hydroxyl Alverine in human plasma. The analytes were extracted from the matrix using a simple solid-phase extraction procedure. Mebeverine was used as the internal standard for both analytes. Akromasil C_8 column provided chromatographic

separation of analytes followed by detection with mass spectrometry. The method involves simple isocratic chromatography conditions and mass spectrometric detection in the positive ionization mode using an API 5000 MS/MS system.

2.2.2. Clinical Methods

2.2.2.1. Poluektova Ye A. et al(2012) reported **"Combined Alverine Citrate and simeticone in preparation in the treatment of irritable bowel syndrome".** To assess the effect of combined drug meteospasmyl on intensity of clinical symptoms of irritable bowel syndrome. Retrospective analysis of efficacy and safety of meteospasmyl in 30 patients with IBS diagnosis, confirmed by conformity of complaints to Rome-III criteria and absence of pathological changes at laboratory and instrumental investigations was carried out.

2.2.2.2. Beyazit et al (2011) reported "**Inclusion of a spasmolytic in bowel cleansing : a prospective randomised study"** The quality of colon cleansing and the tolerance of patients to the procedure are two major determinants of the quality of a colonoscopy. Many bowel cleansing regimens are known, but there is no ideal regimen. Alverine Citrate (Relaxyl, spasmonal) is a spasmolytic agent that has been shown to affect responses of mechanoreceptors of the intestine to both mechanical and chemical stimuli. Patients who underwent colonoscopies at four centres were randomly assigned two different bowel cleansing procedures . The bowel cleansing methods were oral sodium phosphate (NaP) (GroupI) and oral NaP plus Alverine Citrate (Group II). Patients were randomized into one of the regimens. The quality of colon cleansing was assessed by an endoscopist with an empirical, clinically meaningful 3 point scale.

2.2.2.3. Wittmann T. et al (2010) reported " **The Efficacy of Alverine Citrate/Simeticone Combination on Abdominal Pain/discomfort in Irritable**

bowel Syndrome - a randomized, double-blind, Placebo-controlled Study" Alverine citrate and simeticone combination has been used for almost 20 years in irritable bowel syndrome (IBS), but supportive scientific evidence of efficacy was limited. To evaluate the efficacy of Alverine Citrate and simeticone combination in patients with IBS-related abdominal pain/discomfort. A total of 412 IBS patients meeting ROME III criteria were included in this double-blind randomized placebo-controlled study if their abdominal pain/discomfort intensity was at least 60 mm on a 0–100 mm visual analogue scale (VAS) during a 2-week run-in treatment-free period.

2.2.2.4. Donghong ju et al (2009) reported "**Dycolonie and Alverine Citrate enhance the cytotoxic effects of proteasome inhibitor MG 132 on breast cancer cells".** Proteasome is an important target in cancer therapy. To enhance the efficacy of proteaseome inhibitors is a challenging task due to paucity of understanding the functional in mammalian cells. Taking advantage of the knowledge gained from saccharomyces cerevisiae, we show that dyclonine and alverine citrate, the major components of two over – the- counter medicines, can substantially enhance the cytotoxic effects of protesome inhibitor MG132 on breast cancer cells. This study also highlights an important yeast genetic approach to identification of potential therapeutics that can be used for combination therapy with proteasome inhibitors.

2.2.2.5. Engin Altintas et al (2008) reported **"Alverine citrate plus simethicone reduces cecal intubation time in colonoscopy - A randomized study"** Successful colonoscopy depends on the insertion of the instrument to the cecum, a detailed examination, and minimal discomfort to the patient during the procedure. The aim of this study was to determine the effects of alverine citrateplus simethicone on the cecal intubation time, colonicspasm and bowel cleanliness. A prospective, randomized, controlled trial in a consecutive series of patients was conducted to

compare alverine citrate as an antispasmodicagent for relaxation of spasm with elective colonoscopy. The drug used consisted of 60 mg alverine citrate plus 300 mg simethicone. Sodium phosphate soda and enema were recommended for bowel cleansing. During colonoscopy, spasticity, difficulty of the procedure, pain, and cleanliness of the colon were scored between 0-4. The time required to reach the cecum was recorded as minutes.

2.2.2.6. Mehmet Arhanet al (2004) reported "**Alverine citrate induced acute hepatitis"** Alverine citrate is a commonly used smooth muscle relaxant agent. A MEDLINE search on January 2004 revealed only 1 report implicating the hepatotoxicity of this agent. A 34-year-old woman was investigated because of the finding of elevated liver function tests on biochemical screening. Other etiologies of hepatitis were appropriately ruled out and elevated enzymes were ascribed to alverine citrate treatment. Although alverine citrate hepatotoxicity was related to an immune mechanism in the first case, several features such as absence of predictable dose-dependent toxicity of alverine citrate in a previous study and absence of hypersensitivity manifestations in our patient are suggestive of a metabolic type of idiosyncratic toxicity.

2.2.2.7.Mitchell S.A. et al (2002) reported "**Alverine citrate fails to relieve the symptoms of irritable bowel syndrome: results of a double-blind, randomized, placebo-controlled trial"** Alverine citrate has been used in the treatment of irritable bowel syndrome for many years. To compare the efficacy and safety of a new formulation of alverine citrate, a 120-mg capsule, with placebo given three times daily for 12 weeks. One hundred and seven patients with irritable bowel syndrome were entered into this three-centre, double-blind, randomized, placebo-controlled, parallel group trial. The primary end-point was relief of abdominal pain

indicated by improvement in the scores for severity and frequency. Secondary efficacy variables included scores for other clinical symptoms and for overall well-being. The severity and frequency of abdominal pain improved in 66% and 68% of patients treated with Alverine citrate vs. 58% and 69% of the placebo group, but these differences were not significant. The mean percentage reduction in the scores for abdominal pain from baseline to the final assessment, although greater in the Alverine citrate group (43.7%) compared with the placebo group (33.3%), was not statistically significant. Conclusions: Alverine citrate is no better than placebo at relieving the symptoms of irritable bowel syndrome. Future trials should be designed to take into account the high and persistent placebo response seen in this condition.

AIM
AND PLAN OF WORK

3. AIM AND PLAN OF WORK

3.1. AIM OF WORK

The drug analysis plays an important role in the development, manufacture and therapeutic use of drug. Most of the pharmaceutical industries do the quantitative chemical analysis to ensure that the raw material used and the final product thus obtained meet certain specifications and to determine how much of each components are present in the final product.

Standard analytical procedure for newer drugs or formulation may not be available in pharmacopoeias hence it is essential to develop newer analytical methods which are precise, accurate, specific, linear, simple and rapid. UV and HPLC grades of solvents used for respective determination and solvent should readily available and cheaper. The solvent should be completely extracting the active ingredient from formulation.

Alverine citrate is a muscarinic antagonist. It is used for the treatment of smooth muscle relaxant and first approved in the USA. The drug is available in the brand name Spasmonal in US and U.K.

Extensive literature survey revealed that the derivative UV Spectrophotometric method was reported for the estimation of Alverine Citrate in bulk and in formulation. LC-MS/MS method also reported for the detection of Alverine Citrate in plasma.

However there ias no evidence for the estimation of Alverine Citrate by UV spectrophotometry and RP-HPLC in bulk and in capsules formulation. So, an attempt was made to develop simple, cost effective and accurate UV spectrophotometric and RP-HPLC methods for the estimation of Alverine Citrate in bulk and in capsule formulation and to validate the developed methods.

3.2. PLAN OF WORK

3.2.1. Survey on Literature

The survey on literature performed for alverine citrate for their physiochemical properties, solubility, pharmacology and analytical techniques. So this basic information gives notation for newer method development.

3.2.2. Method Development

1) Identification of drug by its melting point and IR spectral studies.

2) Selection of suitable solvents for quantitative extraction of drug present in the formulations. The solvent should be readily available, economical and of analytical grade for UV-spectroscopic method and HPLC grade solvents for RP-HPLC method. The solvent should not interact with the compound of interest and its structural characteristics.

3) Selection of method for analysis, depending on the spectral characteristics of the drug.

4) Development of simple cost effective and accurate RP-HPLC method.

5) Analysis of marketed formulation by the proposed methods.

6) Validation of the development methods as per ICH guidelines. The Parameters used to validate the developed method are Specificity, Linearity, Range, Accuracy, Precision, Limit of Detection, Limit of Quantification, Robustness and Ruggedness

7) Statistical analysis of the developed analytical methods.

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1. MATERIALS USED

4.1.1. Drugs

Alverine Citrate raw material was brought from Meda Pharmaceuticals, US.

4.1.2 Formulation

Spasmonal capsules formulation containing Alverine citrate 120 mg was purchased from US market.

4.1.3. Reagents and Chemicals

All the chemicals and solvents used were of analytical grade and HPLC grade .The chemicals and solvents used for the study were Methanol (HPLC grade), Acetonirile (HPLC grade), ortho phosphoric acid and Water for HPLC, Hydrochloric acid (Analytical grade).

4.1.4. Instruments Used

Different instruments used to carry out the present work are

- Shimadzu AUX- 220 Digital balance.
- Shimadzu- 1700 Double Beam UV-Visible Spectrophotometer with a pair of 10 mm matched quartz cells.
- ELICO SL-210 Double Beam UV- Visible Spectrophotometer with pair of 10 mm matched quartz cells.
- Soltec - Sonica Ultrsonic Cleaner - Model 2200 MH.
- Remi Centrifuge Apparatus
- Cyberlab Micropipette.
- HPLC
- Shimadzu SPD – 20A UV – Visible detector.
- Shimadzu LC – 20 AD solvent delivery module

4.1.5 . Specifications Of Instruments

4.1.5.1. Shimadzu UV - 1700 Double Beam UV Visible Spectrophotometer

Elico - SL 210 Double Beam UV- Visible Spectrophotometer

(**Shimadzu** Instruction Manual; Elico Instruction Manual)

SPECIFICATIONS	
Light source	Tungsten halogen lamp (W), Deuterium lamp (D_2). Light source positions automatic adjustment mechanism
Monochromators	Concave holographic grating with 1200 lines/mm
Detector	Photodiode
Stray light	<0,05%T at 220 nm with NaI 10 g/lt
Measurement Wavelength range	190 to 1100 nm
Spectral Band Width	1.8 nm
Wavelength Accuracy	±0.5 nm
Spectral repeatability	±0.2 nm
Spectral readability	0.1 nm
Recording range	±3.0000 Abs
Photometric accuracy	±0.005 Abs (at 1.0 Abs), ± 0.010 Abs (at 1.5 abs)
Operating temperature/ Humidity	Temperature range: 15 to 35º C Humidity range: 35 to 80% (15 to below 30º C) 35 to 70% (30 to below 35º C)

4.1.5.2. Shimadzu AUX- 220 digital balance (Shimadzu instruction manual)

SPECIFICATIONS	
Weighing capacity	200 gms
Minimum display	0.1 mg
Standard deviation	≤ 0.1 mg
Operating temperature range	5 to 40° C

4.1.5.4. Shimadzu HPLC(Shimadzu Instruction Manual)

Detector Specifications	
Light source	Deuterium arc lamp
Wavelength range	190 to 700 nm
Spectral Band Width	5 nm
Wavelength Accuracy	± 1 nm
Cell path length	10 nm
Cell volume	20 μl
Operating temperature range	4 to 40°C (39 to 104°F)
Recording range	0.0001 to 4.000 AUFS
Operating Temperature/ Humidity	4 to35°C/ 75 %

Pump Specifications	
Pump type	Double reciprocating plunger pump
Pumping methods	Constant flow delivery and constant pressure delivery
Suction filter	45 μm
Line filter	5 μm mesh
Operating temperature	4 to 40°C

4.2. METHODS

In the present work, an attempt was made to develop and validate simple, precise and accurate methods for the estimation of Alverine Citrate in pure and in tablet dosage form by UV-Spectroscopy and RP-HPLC.

4.2.1. UV Spectroscopic Method

4.2.1.1. Selection of solvent

The solubility of Alverine Citrate was determined in variety of solvents as per Indian Pharmacopoeia. Solubility was carried out in different polar and non-polar solvents. From the solubility data 0.1M Hydrochloric acid was selected as solvent for the analysis of Alverine Citrate.

4.2.1.2. Preparation of standard stock solution

40 mg of Alverine citrate standard substance was weighed and transferred into 100 ml volumetric flask, dissolved in 0.1M Hydrochloric acid and made up to the volume with 0.1M Hydrochloric acid. This solution contains 400 μg/ ml concentration.

4.2.1.3. Selection of wavelengths for estimation and stability studies

The standard stock solution was further diluted with 0.1M Hydrochloric acid to get the concentration of 12 μg/ ml and the solution was scanned between 200 - 400 nm using 0.1M Hydrochloric acid as blank. From the spectra, λ max was found to be 207 nm and was selected as an analytical wavelength.

The stability was performed by measuring the solution at different time intervals. It was observed that Alverine Citrate in 0.1M Hydrochloric acid was stable up to 5 hours at the selected wavelength.

4.2.1.4. Preparation of calibration graph

From the standard stock solution 1 – 6 ml were transferred into a series of 100 ml volumetric flasks and made up to the volume with 0.1M Hydrochloric acid. The absorbance of different concentration solutions were measured at 207 nm. The calibration curve was constructed by plotting concentration Vs absorbance. Alverine Citrate was linear with the concentration range of 4 - 24 μg/ ml at 207 nm.

4.2.1.5 . Quantification of raw material

3 ml of standard solution was taken into a series of six 100 ml volumetric flasks and the volume was made up to mark with 01.M Hydrochloric acid. The absorbance of these solutions was measured at 207 nm. The amount of Alverine Citrate present in the raw material was determined by using slope and intercept values from calibration graph.

4.2.1.6. Quantification of formulation

Ten capsules (Spasmonal containing 120 mg of Alverine Citrate) were weighed accurately and the average weight of each capsules was found. The mixed contents of the capsule powder equivalent to 40 mg of Alverine Citrate was weighed accurately and transferred into 100 ml volumetric flask. Added about 60 ml of 0.1M Hydrochloric acid to dissolve the substance and the solution was sonicated for 15 minutes. Then it was made up to the volume to 100 ml with 0.1M Hydrochloric acid (400 μg/ ml) and centrifuged for 15 minutes. The supernatant liquid was filtered through Whatmann filter paper No. 41. From the clear solution, further dilutions were made by diluting 3 ml was pipetted out into a series of six 100 ml volumetric flasks and made up to the mark with 0.1M Hydrochloric acid to get the concentration of 12 μg/ ml of Alverine Citrate theoretically. The absorbance of six replicates were

measured and the amount was calculated by using regression equation. This procedure was repeated for six times.

4.2.1.7. Recovery studies

4.2.1.7.1. Preparation of Alverine Citrate raw material stock solution

160 mg OF Alverine Citrate was accurately weighed and transferred in to 10 ml volumetric flask, sufficient 0.1M Hydrochloric acid was added to dissolve the substance and made up to the mark with the same. This solution contains 16 mg/ ml concentration.

4.2.1.7.2. Recovery procedure

The recovery experiment was done by adding known concentrations of raw material stock solution of Alverine Citrate to the pre analyzed formulation. The capsule powder equivalent to 40 mg of Alverine Citrate was weighed accurately and added 2 ml, 2.5 ml and 3 ml of above raw material stock solution in to a series of 100 ml volumetric flasks, dissolved with 0.1M Hydrochloric acid and sonicated for 15 minutes. The solution was made up to 100 ml with 0.1M Hydrochloric acid and centrifuged for 15 minutes at 2000 rpm. The supernatant liquid was filtered through Whatmann filter paper No. 41. The absorbance of three replicates was measured at the selected wavelength. The amount of drug recovered from formulation was calculated. The procedure was repeated for three times for each concentration.

4.2.1.8. Validation of developed method

4.2.1.8.1. Linearity

A calibration curve was plotted between concentration and absorbance. Alverine Citrate was linear with the concentration range of 4– 24 μg/ ml at 207 nm. The optical characteristics were calculated.

4.2.1.8.2. Precision

The repeatability of the method was confirmed by the analysis of formulation was repeated for six times with the same concentration. The amount of drug present in the capsule formulation was calculated. The percentage RSD and were calculated.

The intermediate precision of the method was confirmed by intraday and inter day analysis. The analysis of formulation was repeated for three times in the same day and one time on three successive days. The amount of drug was determined. Percentage RSD .

4.2.1.8.3. Accuracy

Accuracy of the method was confirmed by recovery studies. To the pre-analyzed formulation, a known quantity of raw material of Alverine Citrate was added and the procedure was followed as per the analysis of formulation. The amount of each drug recovered was calculated. This procedure was repeated for three times for each concentration. The % RSD and confidence were calculated.

4.2.1.8.4. Ruggednes

Ruggedness of the method was confirmed by the analysis of formulation was done by using different instruments and different analysts. The amount was calculated. The % RSD and c were calculated.

4.2.1.8.5. *LOD and LOQ*

The limit of detection (LOD) is the lowest concentration at which the results still satisfy some predetermined acceptance criteria. Below the LOD, the results fail to meet these criteria (analysis is not feasible).

It may expessed

$$LOD = \frac{3.3\sigma}{S}$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

$$LOQ = \frac{10\sigma}{S}$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

4.2.2. REVERSE PHASE – HPLC METHOD

In RP – HPLC, the retention of a compound is determined by its polarity, pKa value, molecular weight, experimental conditions, mobile phase, column and temperature. The column (typically Octyl (C_8) and Octadecyl (C_{18}) bonded phase) is less polar than the water – organic phase, usually an almost or entirely mobile phase. Sample molecules partition between the polar mobile phase and non – polar C_8 and C_{18} stationary phase and more hydrophobic (non - polar) compounds are retained more strongly. Polar compounds are less strongly held and elute from the column first and vice versa. Usually, the lower the polarity of the mobile phase higher in its elution strength. RP – HPLC columns are efficient, stable and reproducible because of the solvents used. Generally gradient and isocratic elution techniques used for elution and an isocratic elution technique employed in this study.

4.2.2.1. METHOD DEVELOPMENT AND OPTIMIZATION OF CROMATOGRAPHIC CONDITIONS

4.2.2.2 Selection of mobile phase and λ max

Solutions of Alverine Citrate 10 μg/ ml was prepared in the mobile phase and scanned in the UV region of 200 – 400 nm and the UV spectrum was recorded. It was found that the λ max of Alverine Citrate in mobile phase was 207 nm. Therefore 207 nm was selected as detection wavelength for the estimation of Alverine Citrate by RP – HPLC with an isocratic elution technique.

4.2.2.3. Stability of sample solutions

Solutions of Alverine Citrate10 μg/ ml were prepared and absorbance was checked for their stability at 207 nm and it was found that drug were stable for 5 hours.

4.2.2.3.1. OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS

4.2.2.3.2 . Initial separation conditions

The following chromatographic conditions were preset initially to get better resolution of Alverine Citrate

Mode of operation	-	Isocratic
Stationary phase	-	C_{18} column (150 mm × 4.6 mm i.d. 5μ)
Mobile phase	-	Acetonitrile:Water(HPLC)
Proportion of mobile phase	-	50: 50% v/v
Detection wavelength	-	207 nm
Flow rate	-	1ml/ min
Temperature	-	Ambient
Sample load	-	20 μl
Method	-	External Standard Calibration method.

The mobile phase was primarily allowed to run for 30 minutes to record a steady baseline. The solution of Alverine Citrate was injected and the respective chromatogram was recorded. It was found that Alverine Citrate were eluted with broad peak. For this reason, different ratios of mobile phase with different solvents were tried to obtain good chromatogram with acceptable system suitability parameters.

S.No	Mobile phase	Observation
1	Acetonitrile : Water (50: 50% v/v)	Broad peak was obtained
2	Acetonitrile: Water pH adjusted to 3.5 with orthophosphoric acid (50: 50% v/v)	Broad peak with tailing was obtained
3	Acetonitrile: Water pH adjusted to 3.5 with orthophosphoric acid (70: 30% v/v)	Peak splitting was obtained
4	Acetonitrile: Water pH adjusted to 3.5 with orthophosphoric acid (60:40% v/v)	Peak was sharp but splitting was obtained
4	Acetonitrile: Phosphate buffer pH adjusted to 3.5 with orthophosphoric acid	Sharp peak was obtained

With the above information's, Acetonitrile: Phosphate buffer and pH adjust to 3.5 of with orthophosphoric acid was selected as mobile phase and further optimization was done with this mobile phase.

4.2.2.3.3 OPTIMIZED OF CHROMATOGRAPHIC CONDITIONS

The following optimized chromatographic conditions were employed for the analysis of Alverine Citrate by isocratic RP – HPLC method.

Mode of operation	-	Isocratic
Stationary phase	-	C_{18} column (150 mm × 4.6 mm i.d. 5μ)
Mobile phase	-	Acetonitrile : Phosphate buffer pH 3.7 adjusted with orthophosphoric acid .
Proportion of mobile phase	-	60:40 % v/ v
Detection wavelength	-	207 nm
Flow rate	-	1ml/ min
Temperature	-	Ambient
Sample load	-	20 μl
Operating pressure	-	140kgf
Method	-	External Standard Calibration method.

4.2.2.3.2 . Preparation of standard Alverine Citrate solution

50 mg of Alverine Citrate was weighed accurately and transferred into 50 ml volumetric flask, dissolved in mobile phase and the volume was made up to the mark with mobile phase. Further, 2.5 ml of mother liquor was pipetted into 25 ml volumetric flask and the volume was made with mobile phase to acquire 100 μg/ ml concentration.

4.2.2.3.3. Preparation of Calibration graph

In this progression, the aliquots of stock solution of Alverine Citrate (1-6 ml) was transferred into a series of 10 ml volumetric flasks and made up to the mark with mobile phase. The solutions containing the concentrations of 10- 60 μg/ ml of Alverine Citrate. The solutions were injected and the chromatograms were recorded.

The above concentration range was found to be linear and obeys Beer's law. The procedure was repeated for three times. The peak areas were plotted against concentration and the calibration curve was constructed.

4.2.2.3.4. Estimation of Alverine Citrate capsule formulation

Ten capsules of formulation (Spasmonal containing 120 mg of Alverine Citrate) was weighed accurately. The average weight of each capsule was found and powdered. The mixed contents of the capsule powder equivalent to 50 mg of Alverine Citrate was weighed accurately and transferred into 50 ml volumetric flask. Added about 30 ml of mobile phase to dissolve the substance and made up to the volume with the same (1000 µg/ ml). The solution was sonicated for 15 minutes, centrifuged at 2000 rpm for 15 minutes and filtered through Whatmann filter paper No. 41. From the clear solution, further dilutions were made by diluting 2.5 ml into 25 ml with mobile phase to obtain 100 µg/ ml concentration.

4.2.2.3.5. Assay Procedure

2 ml of test solution was transferred into six 10 ml volumetric flasks individually and made up to the mark with mobile phase to obtain 20 µg/ ml. A steady base line was recorded with optimized chromatographic conditions. After stabilization, the test solutions were injected and recorded the chromatograms. The concentration of each test solution was determined by using slope and intercept values from the calibration graph.

4.2.2.3.6. Recovery Experiments

a) Preparation of Alverine Citrate raw material stock solution

An accurately weighed quantity of 40 mg of Alverine Citrate was transferred into 10 ml volumetric flask and added sufficient mobile phase to dissolve the

substance and made up to the mark with the same. This contains 4 mg/ ml concentration.

b) Procedure

The recovery experiment was done by adding known concentrations of raw material stock solution of Alverine Citrate to the pre analyzed formulation. The capsules powder equivalent to 50 mg of Alverine Citrate was weighed accurately and added 2 ml, 2.5 ml, and 3 ml of raw material stock solution into 50 ml standard flasks individually and dissolved in mobile phase and made up to the mark with same. The solution was sonicated for 15 minutes. After sonication, centrifuged for further 15 minutes at 2000 rpm and the supernatant liquid was filtered through Whatmann filter paper no 41. 2.5 ml of the solution was diluted to 25 ml with mobile phase. further, 2 ml of the solution was made up to 10 ml with mobile phase. The solution was injected and the chromatograms were recorded. The amount of drug recovered was calculated by using slope and intercept valves from the calibration graph.

LOD and LOQ

The linearity study was carried out for three times. The LOD and LOQ were calculated based upon the calibration curve method. The LOD and LOQ were calculated using the average of slope and standard deviation of intercept.

System suitability studies

The system suitability studies conceded as per ICH guidelines and USP. The parameters like tailing factor, asymmetry factor, capacity factor, number of theoretical plates and HETP were calculated.

RESULTS AND DISCUSSION

5. RESULTS AND DISCUSSION

The following methods were developed and validated for the estimation of Alverine Citrate in pure and in capsule dosage form. The methods were

1) UV spectroscopic method
2) RP-HPLC method

5.1. UV SPECTROSCOPIC METHOD

Alverine Citrate was identified by using melting point analysis and IR spectral studies (figure1). The solubility of Alverine Citrate was determined as per Indian Pharmacopoeia. The number of polar and non - polar solvents were tried to dissolve the drug. From the solubility data, Alverine Citrate is freely soluble in Methanol, Ethanol, ethyl acetate, Dimethyl formamide, Glacial Acetic Acid, alkaline borate buffer, isopropyl alcohol, 0.1M Hydrochloric acid and Acetonitrile. It is sparingly soluble in Acetone, Chloroform, phosphate Buffer pH-7 and Dichloro methane. Slightly soluble in Diethyl ether and Carbon tetra chloride; very slightly soluble in Distilled water, Acid Phthalate buffer pH3 and Acid phthalate buffer pH 2. Alverine Citrate is practically insoluble in 0.1M Sodium hydrochloride, Benzene, Toluene, Neutralized Phthalate buffer pH 5. From the solubility profile 0.1M Hydrochloric acid was selected as the solvent because of it is cheap and easily available. The solubility profile of Alverine Citrate is given in table 1.

The spectrum of Alverine Citrate was recorded in the wavelength range 200 – 400 nm. From the spectrum, the maximum absorbance was observed at 207 nm. Hence 207 nm was selected for the analysis of Alverine Citrate. This is shown figure 2. The stability of Alverine Citrate was studied by measuring the absorbance at different time intervals. It was observed that the drug was stable up to 5 hours in 0.1M Hydrochloride acid.

Different aliquots of Alverine Citrate was prepared in the concentration range of 4 to 24 µg/ ml. The absorbance of solutions were measured at 207 nm. The calibration curve was plotted using concentration against absorbance. The preparation of calibration curve was repeated for six times. The optical characteristics like Correlation coefficient, Slope, Intercept, Molar Absorptivity, Sandell's sensitivity, LOD and LOQ were calculated. The correlation coefficient value for the calibration graph was found to be 0.9995. This indicates that the absorbance was linear with the concentration range of 4 – 24 µg/ ml. The calibration curve of Alverine Citrate is shown in figure 3. The optical characteristics are listed in table 2.

To confirm the developed method, quantification of raw material was done and the amount was calculated. The percentage amount of Alverine Citrate was found to be 99.99 ± 0.0734. The amount found by this method was close to 100%. Hence this method can be applied for the analysis of formulation. The results are shown in table 3.

Spasmonal capsules was selected for analysis. The nominal concentration of Alverine Citrate from the linearity was prepared and the absorbance of the solution was measured at 207 nm. The amount of test solution was calculated by using slope and intercept values. This procedure was repeated for six times to ensure the precision of the method. The percentage label claim in capsules formulation was found to be 98.25.% ± 1.0034. The amount present in tablet formulation was good concord with the label claim and the Percentage Relative Standard Deviation (% RSD) value was found to be 1.0213. The low % RSD value indicates that the method has good precision. The results of analysis are shown in table 4.

The intermediate precision of the method was confirmed by intraday and inter day analysis. The analysis of formulation was repeated for three times in the same day

and one time in the three successive days. The percentage RSD value for intraday and inter day analysis of Alverine Citrate was found to be 0.7511 and 0.9475, respectively. The reports of analysis are shown in tables 5 and 6. The results showed that the intermediate precision of the method was confirmed.

The developed method was validated for Ruggedness. It refers to the analysis should be done in same laboratory which may include multiple analysts, multiple instruments and different sources of the reagents and so on. . The percentage RSD value for analyst 1 and analyst 2 were found to be 0.0734 and 0.2349 respectively. The % RSD value for instrument 1 and instrument 2 were found to be 0.0734 and for 0.5681, respectively. The low % RSD values indicate that the method was more rugged. Reports of analysis are shown in tables 7 and 8 for different analyst and different instruments, respectively.

The accuracy of the method was confirmed by recovery studies. To the pre-analyzed formulation a known quantity of Alverine Citrate raw material solution was added at three different concentrations. The concentration of standard raw material added were 80%, 100% and 120% of the sample concentration. The absorbance of the solutions was measured and the percentage recovery was calculated. The percentage recovery was found to be in the range of 102.56 to 100.20 of Alverine Citrate. The % RSD was found to be less than 2. The low % RSD values indicated that there are no interferences due to excipients used in formulation during the analysis of Alverine Citrate from capsule formulation. Hence the method was found to be accurate. The recovery data were shown in table 9.

5.22. RP – HPLC METHOD

An exertion has been made to simple, precise, rapid, specific and accurate method for the estimation of Alverine citrate in pure form and in formulation by RP – HPLC method.

The solution of 10 μg/ ml of Alverine Citrate in mobile phase (Acetonitrile: Phosphate buffer 3.5 adjusted with orthophosphoric acid in the proportions of 60: 40% v/v) was prepared and the solution was scanned in the range of 200 – 400 nm. At 207 nm the drug showed maximum absorbance. Hence, 207 nm was selected as detection wavelength for the estimation of Alverine Citrate by RP – HPLC method with isocratic elution technique and it was found that Alverine Citrate was stable up to 5 hours.

Based up on the properties of the drug, the initial separation was achieved by using different mobile phase with different compositions. The mobile phase consists of Acetonitrile: Water (50: 50% v/v) was initially tried and chromatograms was recorded. This is shown in figure 4. At 50: 50% v/v ratio, the drug was eluted with broad peak. Further Acetonitrile : Water (pH adjusted to 3.5 with orthophosphoric acid) as mobile phase was tried in different ratios. The ratio tried were 50:50% v/v 60:40% v/v and 70:30% v/v. In all the ratio peak splitting and tailing was occur. Hence it was planned to change phosphate buffer instead of water.The chromatograms are shown in figure 5, 6 and 7 respectively. Mobile phase consisting of Acetonitrile: Phosphate buffer pH adjusted to 3.5 with orthophosphoric acid in the ratio of 60:40 v/v was tried. A sharp peak was obtained with marked retention time. Hence this was selected as mobile phase. The optimized chromatogram is shown in figure 8. The system suitability parameter were calculated and are shown in table 10.

With the optimized chromatographic conditions, stock solutions of Alverine Citrate were prepared by using mobile phase in the concentrations in the range of 10 - 60 μg/ ml. Each solution was injected and chromatograms were recorded. The chromatograms are shown in figures 9 - 14.

The calibration curve was plotted using concentration against peak area. The procedure was repeated for three times. The correlation co-efficient value was found to be 0.9999. The calibration graph is shown in figure 15. The optical characteristics like correlation coefficient, slope, intercept, LOD, LOQ were calculated and are shown in table 11.

The capsules formulation (spasmonal) was selected for the analysis. The nominal concentration (20 μg/ ml) from calibration curve was prepared in mobile phase. The formulation was injected and the chromatogram was recorded. The precision of the method was confirmed by repeatability of formulation for six times and chromatograms were recorded as shown in figures 16 - 21. The percentage purity of Alverine Citrate present in formulation was found to be 100.06 ± 0.2186 The % RSD value was found to be 0.2815. It indicates that the method has good precision. The values are shown in table 12.

The accuracy of the method was performed by recovery studies. To the preanalyzed formulation a known quantity of Alverine Citrate raw material solution was added at three different concentrations (80%, 100% and 120%) and solution were injected. The chromatograms were recorded and are shown in the figures 22 - 30. The percentage recovery was found to be in the range of 99.91 - 101.69 of Alverine Citrate. The % RSD values were found to be less than 2 for all the concentrations. The Low % RSD values indicated that there are no interferences due to the excipients used

in formulation during the analysis of alverine citrate from capsules formulation. Hence the method was found to be accurate. The recovery data is shown in table 13.

SUMMARY AND CONCLUSION

6. SUMMARY AND CONCLUSION

A simple, rapid, precise and accurate UV spectrophotometric method and an RP-HPLC method were developed and validated for estimation of Alverine Citrate in pure form and in capsule dosage form.

The methods employed for the analysis of Alverine Citrate were

1) UV spectroscopic method
2) RP-HPLC method

6.1. UV SPECTROSCOPIC METHOD

From the solubility data the solvent selected for solubility was found to be 0.1M Hydrochloric acid. The λ max of Alverine Citrate in 0.1M Hydrochloride was 207 nm. Alverine Citrate was linear with the concentration range of 4- 24 µg/ ml. The correlation coefficient value for the calibration graph was found to be 0.9995. The percentage of Alverine Citrate present in the prepared raw material solution was found to be 99.99 ± 0.0734. Spasmonal containing 120 mg of Alverine Citrate were selected for analysis. The percentage label claim present in the capsules formulation was found to be 98.25 ± 1.0034 of Alverine Citrate. The precision of the method was confirmed by the repeated analysis of formulation. The %RSD was found to be 1.0213.

Further precision of the method was confirmed by intraday and inter day analysis. The percentage RSD value of the intraday and inter day analysis of Alverine Citratewas found to be 0.7511 and 0.9475, respectively. The developed method was validated for ruggedness. The percentage RSD value by analyst 1 and analyst 2 were found to be 0.0734 and 0.2349, respectively and by instrument 1 and instrument 2 were found to be 0.0734 and for 0.5681 respectively. The low % RSD values indicate that the developed method was more rugged. The accuracy of the method was

confirmed by recovery studies. The percentage recovery was found to be in the range of 102.56 to 103.20% of Alverine Citrate.

6.2 RP-HPLC METHOD

An exertion has been made for a simple, precise, rapid, specific and accurate method for the estimation of Alverine Citrate in pure form and in formulation by RP – HPLC method. After optimizing the mobile phase, with the consideration of the system suitability parameters, Acetonitrile: phosphate buffer (pH adjusted to 3.5 with orthophosphoric acid) in the ratio of 60: 40% v/v was selected as mobile phase for the analysis.

With the optimized chromatographic conditions, the drug was linear in the concentration range of 10 – 60 μg/ ml. the correlation coefficient was found to be 0.9999.

The percentage purity of Alverine citrate present in formulation was found to be 100.06 ± 0.2186. The precision of the method was confirmed by repeatability of formulation for six times.

The accuracy of the method was performed by recovery studies. The percentage recovery was found to be in the range of 99.91 to 101.69% of Alverine Citrate. The % RSD value was found to be 08450. It the low % RSD values for recovery indicated that the method was found to be accurate.

A simple, rapid and accurate UV – spectroscopy method and an isocratic RP – HPLC method were developed for the determination of Alverine Citrate in bulk and in capsule . The methods showed excellent sensitivity, reproducibility, accuracy and repeatability, which is evidenced by low percentage relative standard deviation values. The results obtained in recovery studies were indicating that there is no interference from the excipients used in the formulation. By comparing two methods,

UV - spectroscopy method was found to be economic when compared to RP - HPLC. Because the solvents and column used in RP - HPLC are very costly. When comparing the sensitivity of the methods, RP - HPLC method was found to be more sensitive than UV - spectroscopy method. Because the linearity range, LOD and LOQ were less in RP - HPLC method than UV – spectroscopic method. Hence it is suggested that the proposed UV spectroscopic methods and RP - HPLC method can be effectively applied for the routine quality control analysis of Alverine Citrate in bulk and in capsule formulation.

FIGURES

FIGURE – 1
IR SPECTRUM OF ALVERINE CITRATE

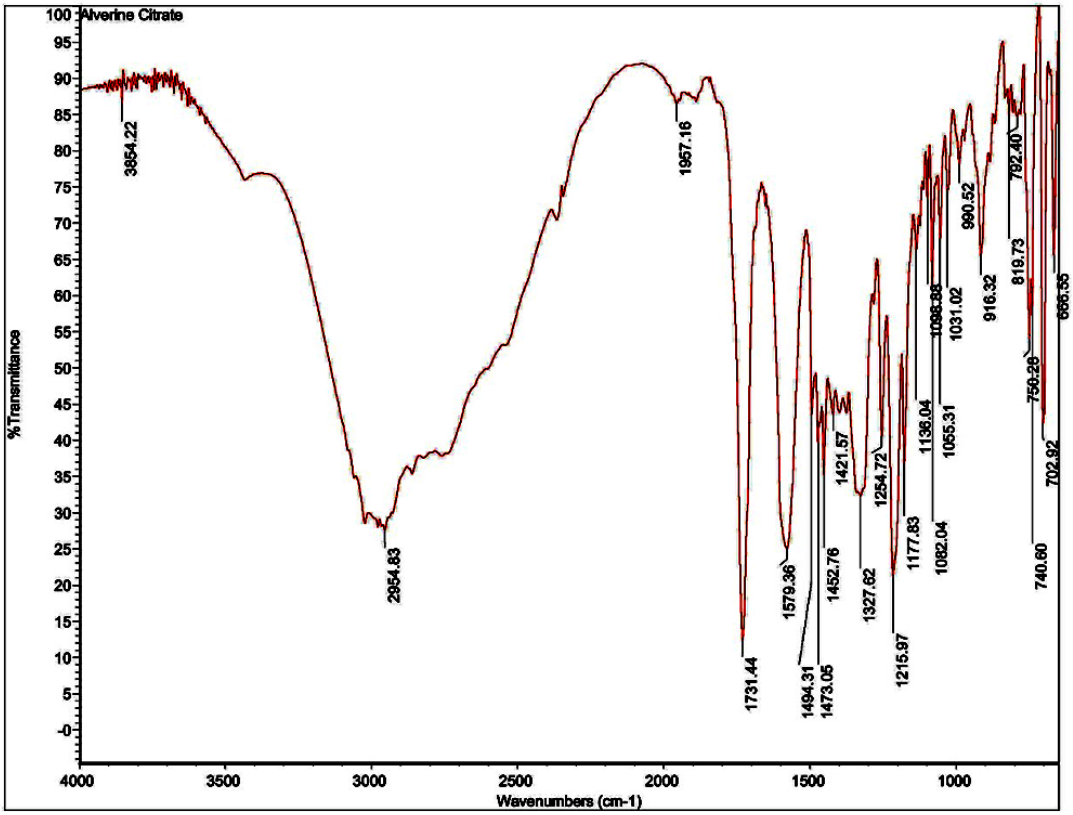


FIGURE – 2
UV SPECTRUM OF ALVERINE CITRATE IN 0.1M HYDRO CHLORIC ACID(10 µg/ ml)

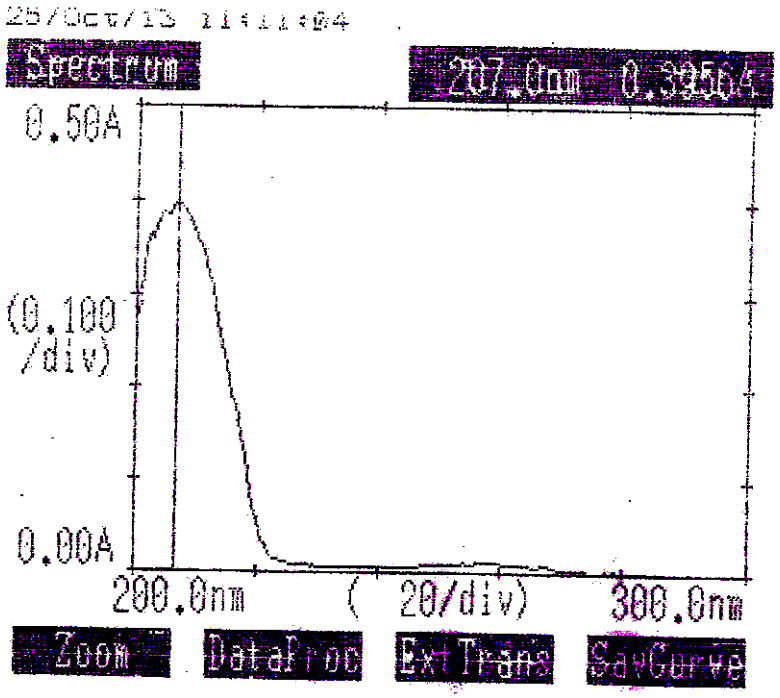


FIGURE – 3
CALIBRATION CURVE OF ALVERINE CITRATE IN 0.1M HYDRO CHLORIC ACID AT 207 nm BY UV SPECTROSCOPY

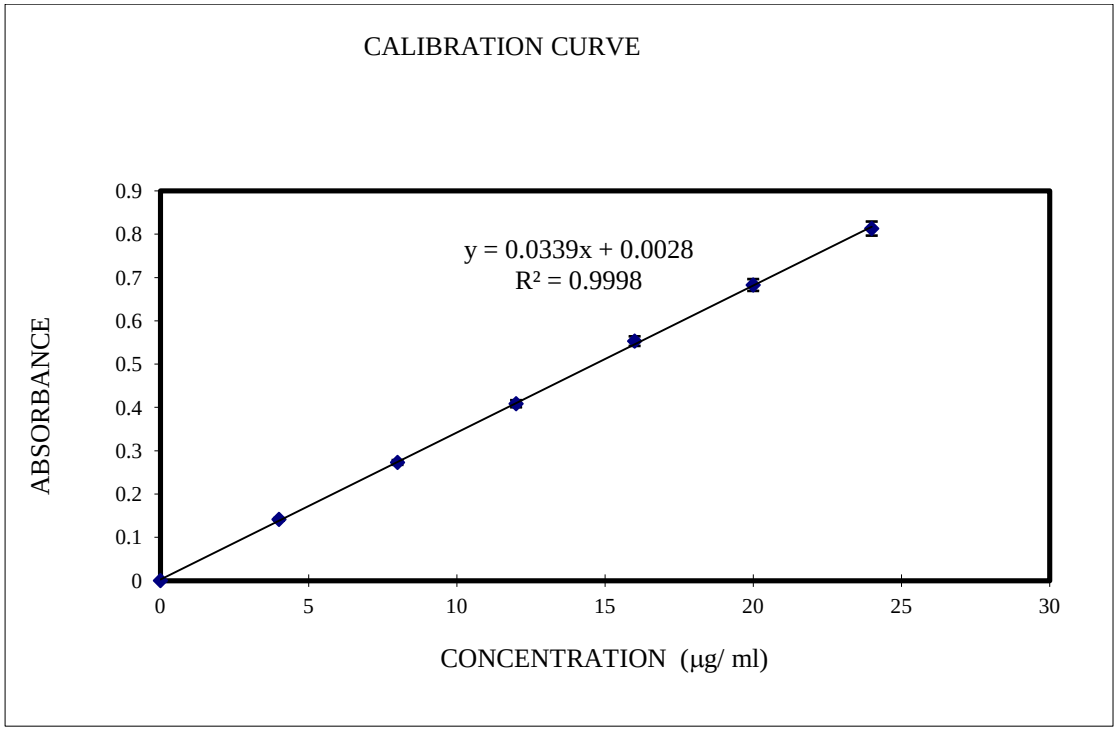


FIGURE – 4
INITIAL SEPERATION CONDITIONS
ACETONITRILE: WATER (50:50% v/v)

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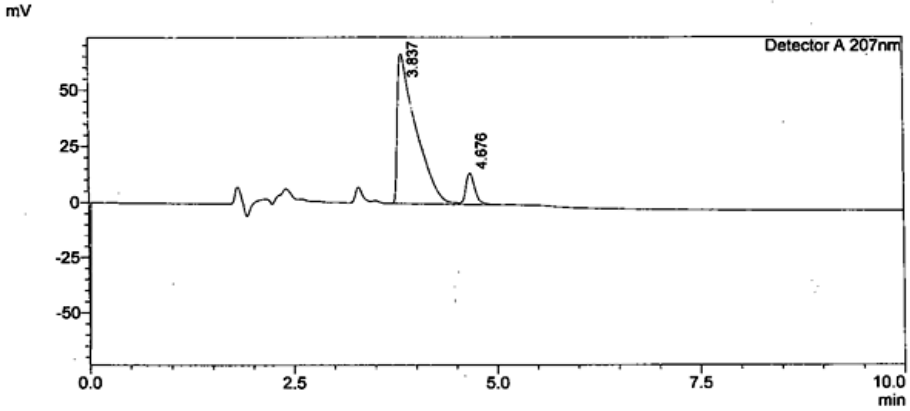
SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

Sample Name	: ALVERINE CITRATE		
Sample ID	: ALVERINE CITRATE		
Data Filename	: ALV-02.lcd		
Method Filename	: ALV.lcm		
Batch Filename	:		
Vial #	: -1	Sample Type	: Unknown
Injection Volume	: 20 uL		
Date Acquired	: 1/22/2014 3:28:33 PM	Acquired by	: user1
Date Processed	: 1/22/2014 3:38:34 PM	Processed by	: user1

<Chromatogram>



<Peak Table>

Detector A 207nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	3.837	1063692	66704	90.603			
2	4.676	110316	13934	9.397		V	
Total		1174008	80638				

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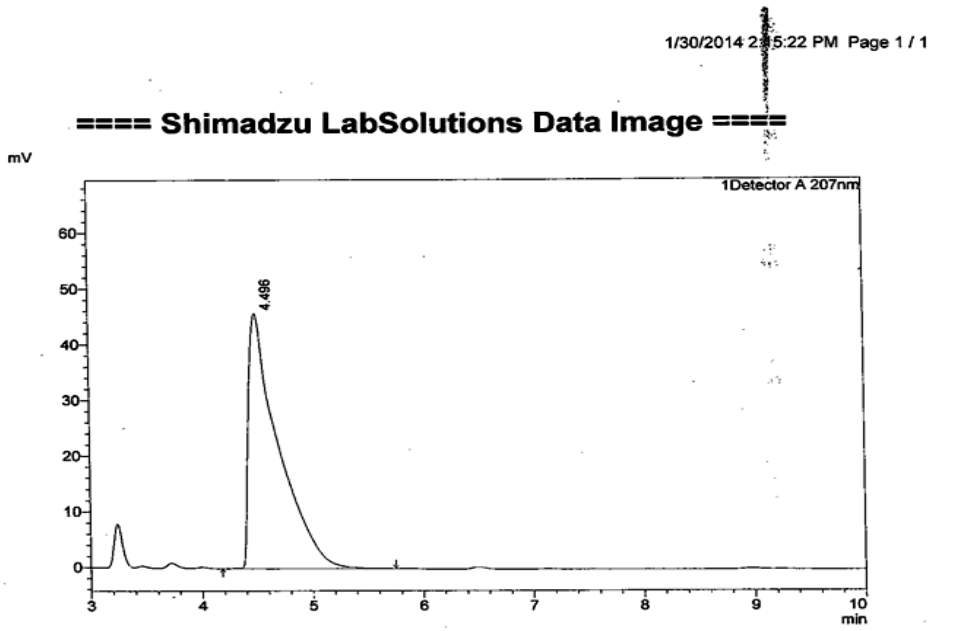
FIGURE-5
INITIAL SEPERATION CONDITIONS
ACETONITRILE: WATER pH 3.5 (50:50% v/v)



Detector A 207nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	4.496	860893	45746	100.000		S
Total		860893	45746			

Name

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FIGURE - 6
INITIAL SEPERATION CONDITIONS
ACETONITRILE: WATER PH 3.5 (60:40% v/v)

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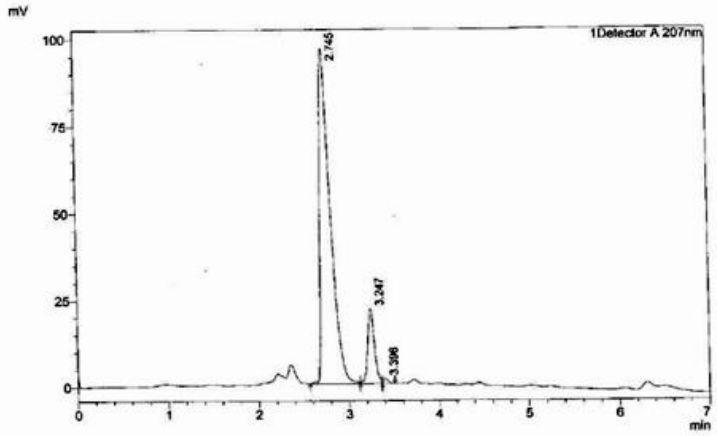
==== Shimadzu LabSolutions Data Image ====



Detector A 207nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	2.745	774520	96405	85.782		
2	3.247	111860	21433	12.532		V
3	3.396	6124	1490	0.686		V
Total		892604	119330			

Name

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FIGURE - 7
INITIAL SEPERATION CONDITIONS ACETONITRILE: WATER PH 3.5 (70:30% v/v)

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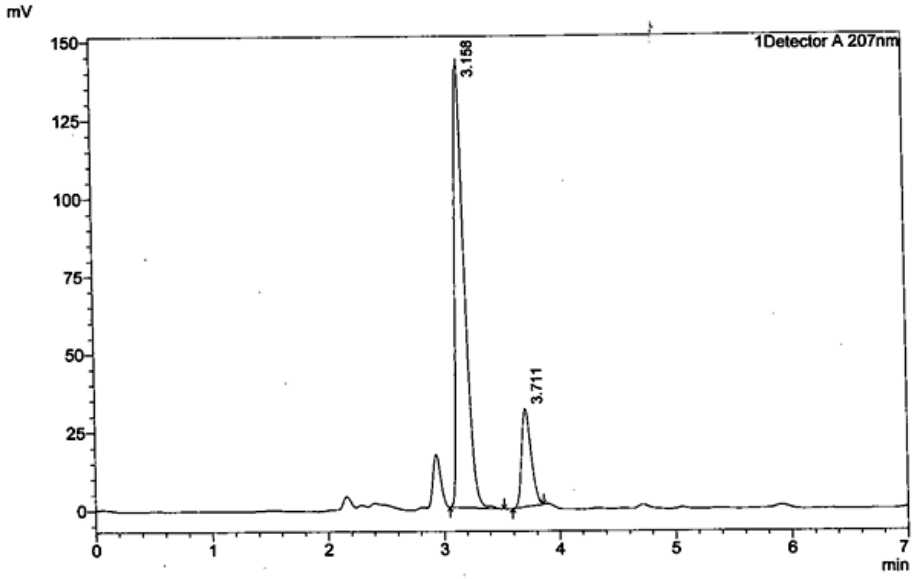
==== Shimadzu LabSolutions Data Image ====



Detector A 207nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.158	813780	143567	79.216		
2	3.711	176652	31168	17.196		
3	7.027	6949	733	0.676		V
4	7.445	3865	255	0.376		V
5	8.471	4430	403	0.431		V
6	9.042	2495	148	0.243		V
7	9.444	19127	1773	1.862		V
Total		1027297	178046			

Name

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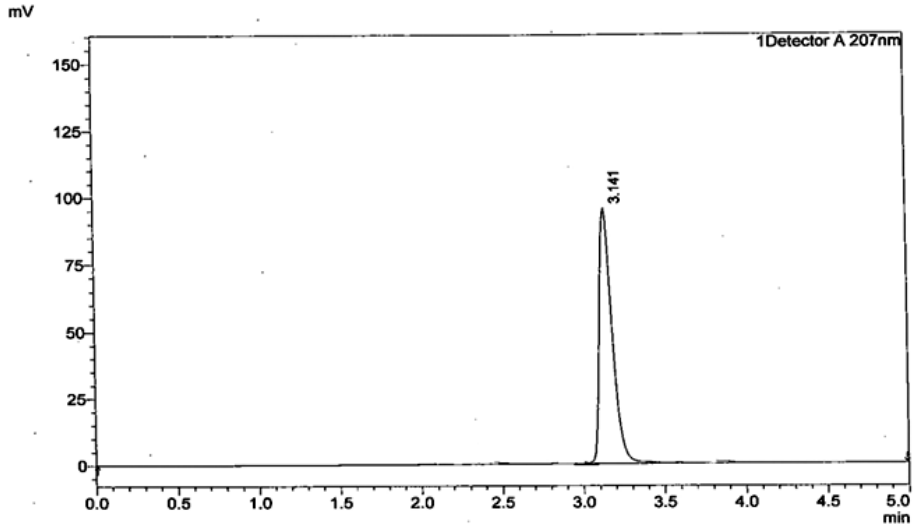
FIGURE-8
OPTIMIZED CHROMATOGRAM

==== Shimadzu LabSolutions Data Image ====



Detector A 207nm

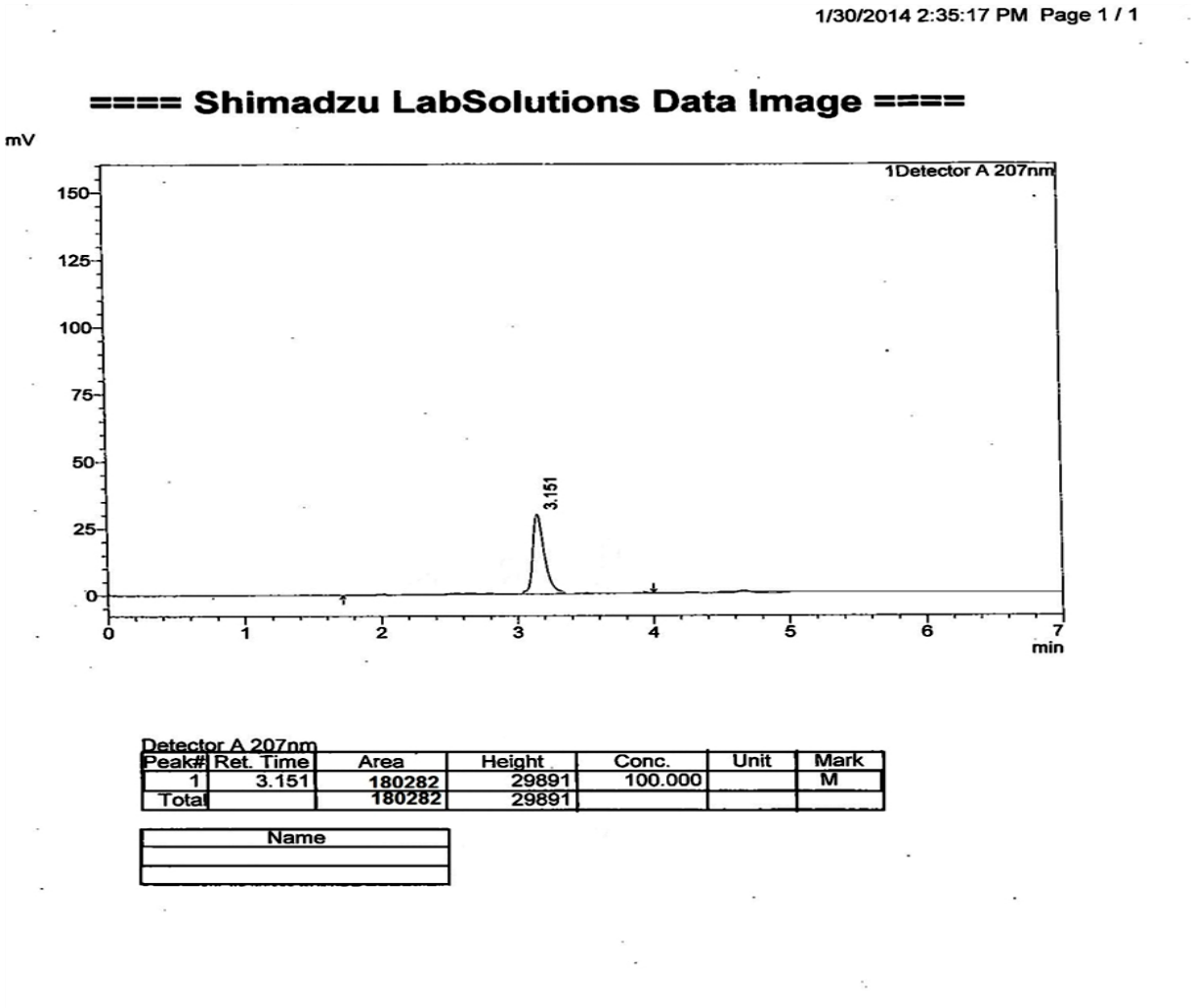
Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.141	541432	95197	100.000		M
Total		541432	95197			

Name

E:\2012 - 13\2013-14\ALV\ALV-29.lcd

FIGURE – 9
LINEARITY CHROMATOGRAM OF ALVERINE CITRATE

(10µg/ ml)



Detector A 207nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.151	180282	29891	100.000		M
Total		180282	29891			

Name

FIGURE – 10
LINEARITY CHROMATOGRAM OFALVERINE CITRATE
(20 µg/ml)

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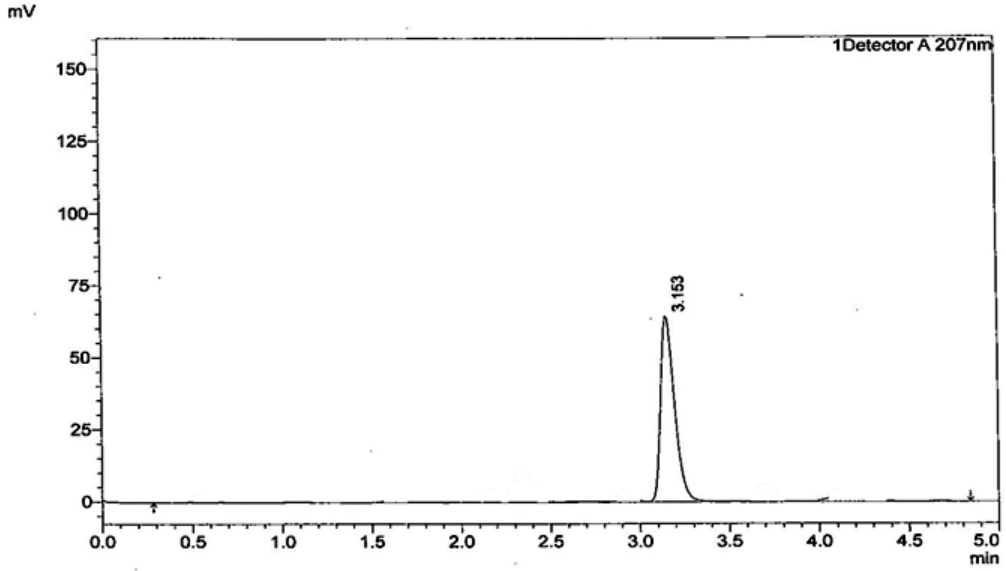
==== Shimadzu LabSolutions Data Image ====



Detector A 207nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.153	360423	64168	100.000		M
Total		360423	64168			

Name

E:\2012 - 13\2013-14\ALV\ALV-23.lcd

FIGURE –11
LINEARITY CHROMATOGRAM OF ALVERINE CITRATE
(30 µg/ ml)

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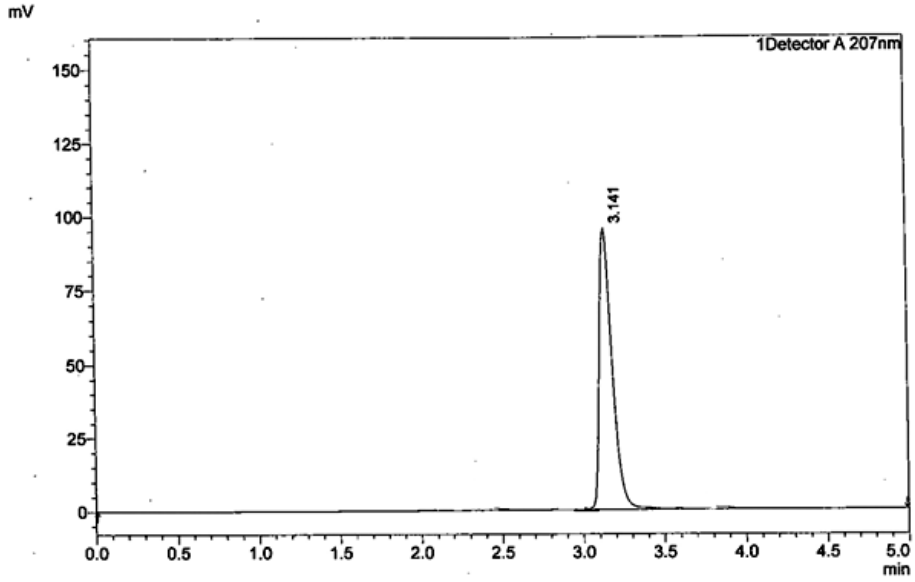
==== Shimadzu LabSolutions Data Image ====



Detector A 207nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.141	541432	95197	100.000		M
Total		541432	95197			

Name

E:\2012 - 13\2013-14\ALV\ALV-29.lcd

FIGURE – 12
LINEARITY CHROMATOGRAM OF ALVERINE CITRATE
(40 µg/ ml)

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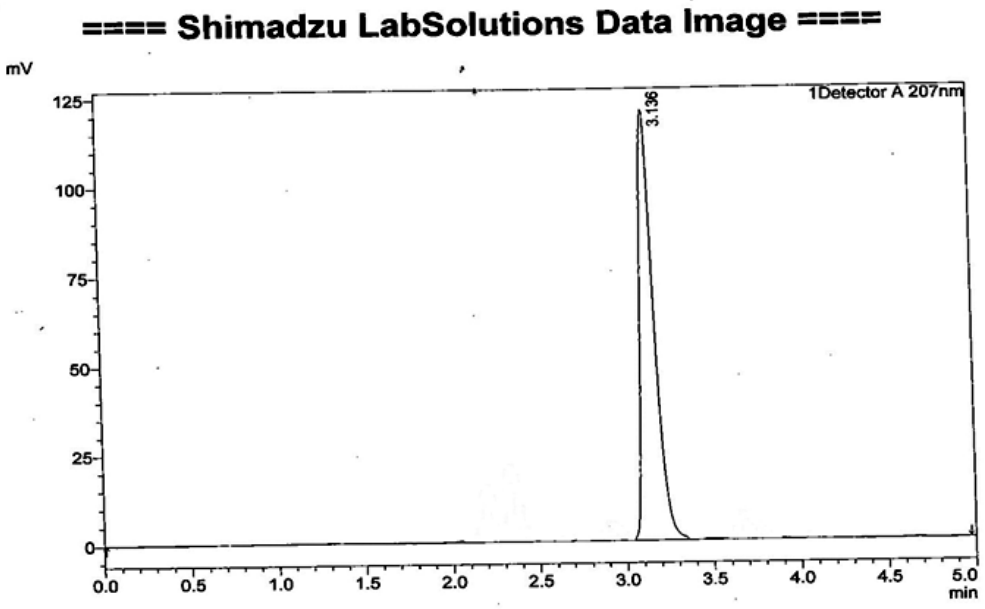

Detector A 207nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.136	720654	120704	100.000		M
Total		720654	120704			

Name

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FIGURE – 13
LINEARITY CHROMATOGRAM OF ALVERINE CITRATE
(50 µg/ ml)

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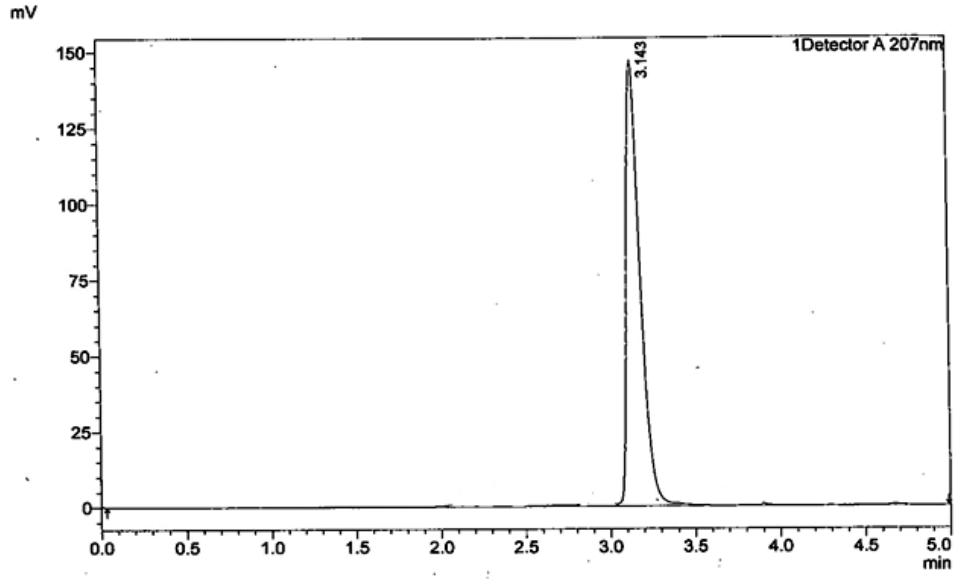
==== Shimadzu LabSolutions Data Image ====



Detector A 207nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.143	912543	146973	100.000		M
Total		912543	146973			

Name

E:\2012 - 13\2013-14\ALV\ALV-37.lcd

FIGURE – 14
LINEARITY CHROMATOGRAM OF ALVERINE CITRATE
(60 µg/ ml)

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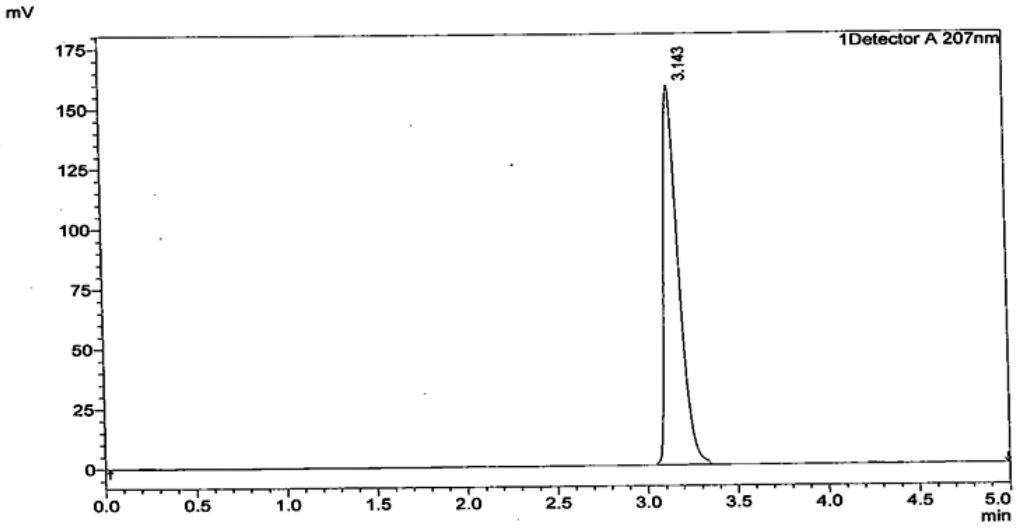
==== Shimadzu LabSolutions Data Image ====



Detector A 207nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.143	1090567	158640	100.000		M
Total		1090567	158640			

Name

E:\2012 - 13\2013-14\ALV\ALV-39.lcd

FIGURE – 15
CALIBRATION CURVE OF ALVERINE CITRATE BY RP-HPLC

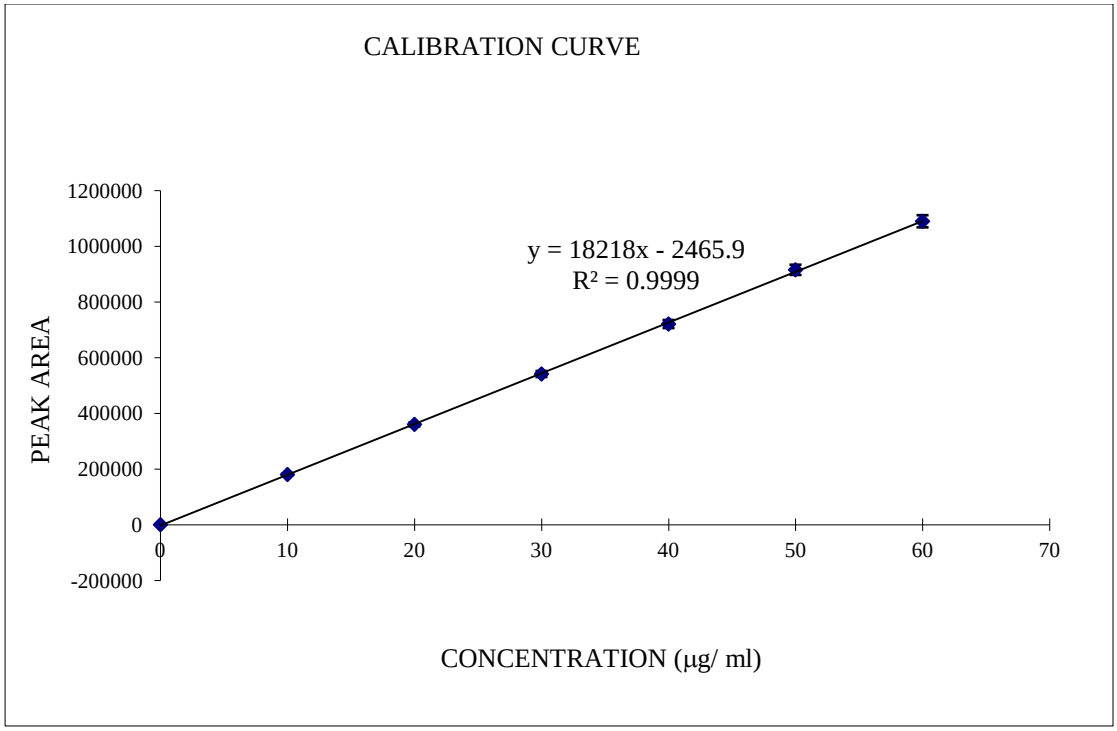


FIGURE – 16
REPEATABILITY ANALYSIS OF FORMULATION - 1
BY RP- HPLC

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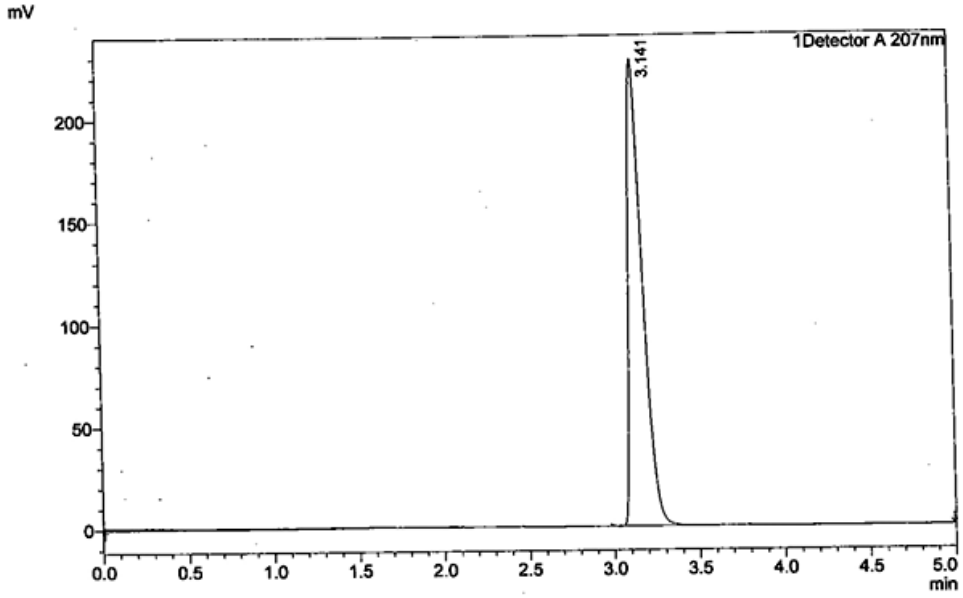
==== Shimadzu LabSolutions Data Image ====



Detector A 207nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.141	361324	228233	100.000		M
Total		361324	228233			

Name

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FIGURE –17
REPEATABILITY ANALYSIS OF FORMULATION - 2
BY RP- HPLC

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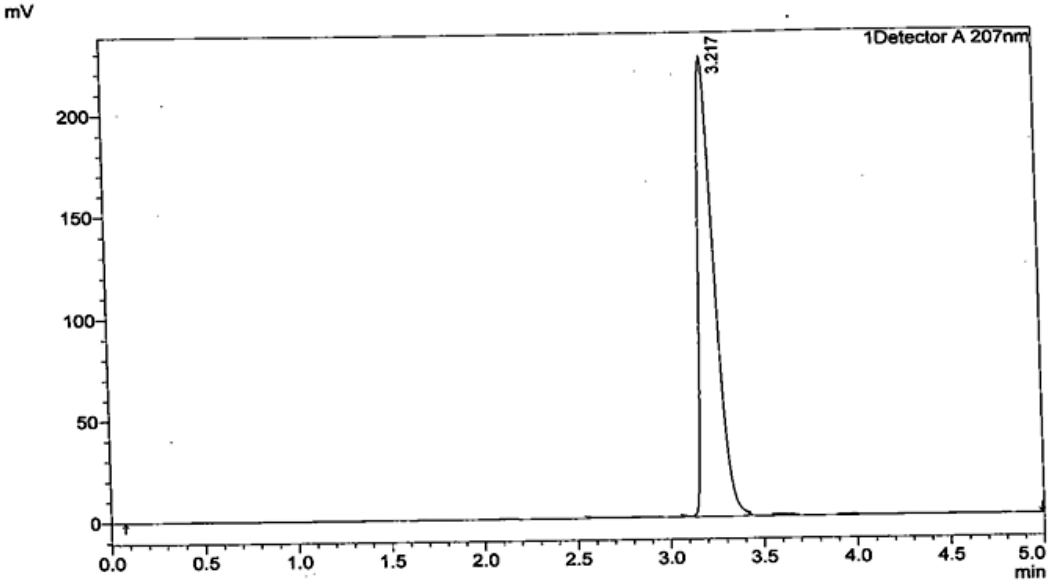
==== Shimadzu LabSolutions Data Image ====



Detector A 207nm

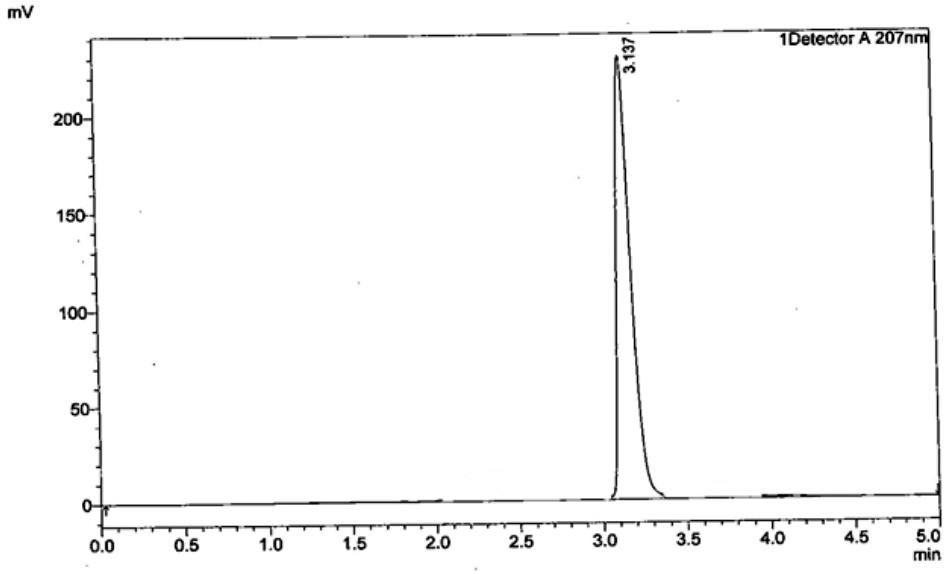
Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.217	361324	226519	100.000		M
Total		361324	226519			

Name

E:\2012 - 13\2013-14\ALV\ALV-42.lcd

FIGURE – 18
REPEATABILITY ANALYSIS OF FORMULATION - 3
BY RP- HPLC

==== Shimadzu LabSolutions Data Image ====



Detector A 207nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.137	362642	229414	100.000		M
Total		362642	229414			

Name

E:\2012 - 13\2013-14\ALV\ALV-43.lcd

FIGURE – 19
REPEATABILITY ANALYSIS OF FORMULATION - 4
BY RP- HPLC

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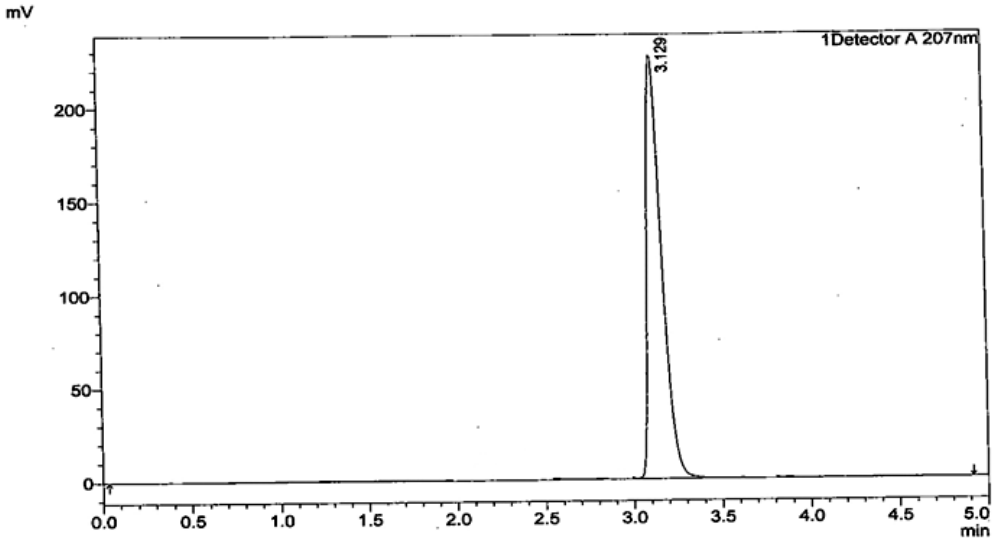
==== Shimadzu LabSolutions Data Image ====



Detector A 207nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.129	361456	227239	100.000		M
Total		361456	227239			

Name

E:\2012 - 13\2013-14\ALV\ALV-45.lcd

FIGURE-20
REPEATABILITY ANALYSIS OF FORMULATION - 5
BY RP- HPLC

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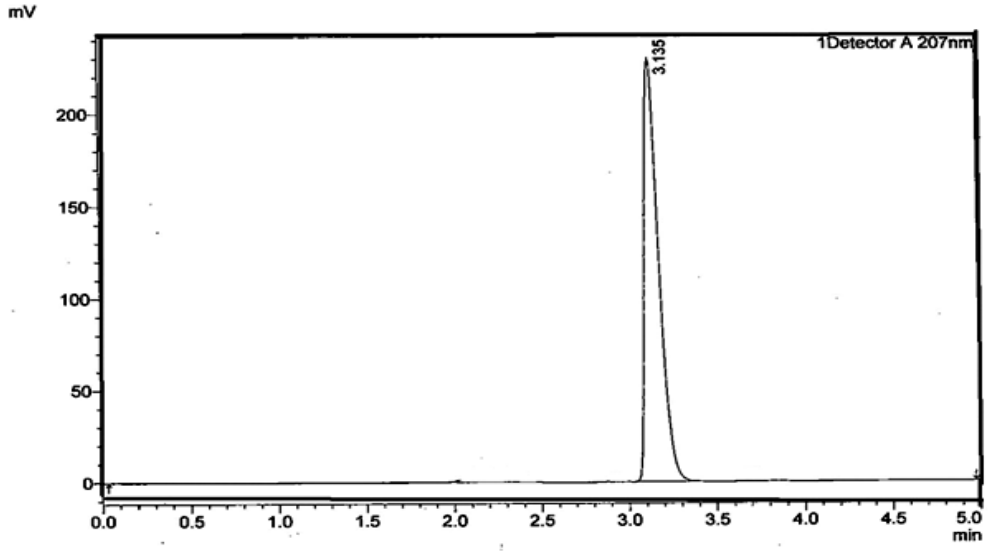
==== Shimadzu LabSolutions Data Image ====



Detector A 207nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.135	363214	230453	100.000		M
Total		363214	230453			

Name

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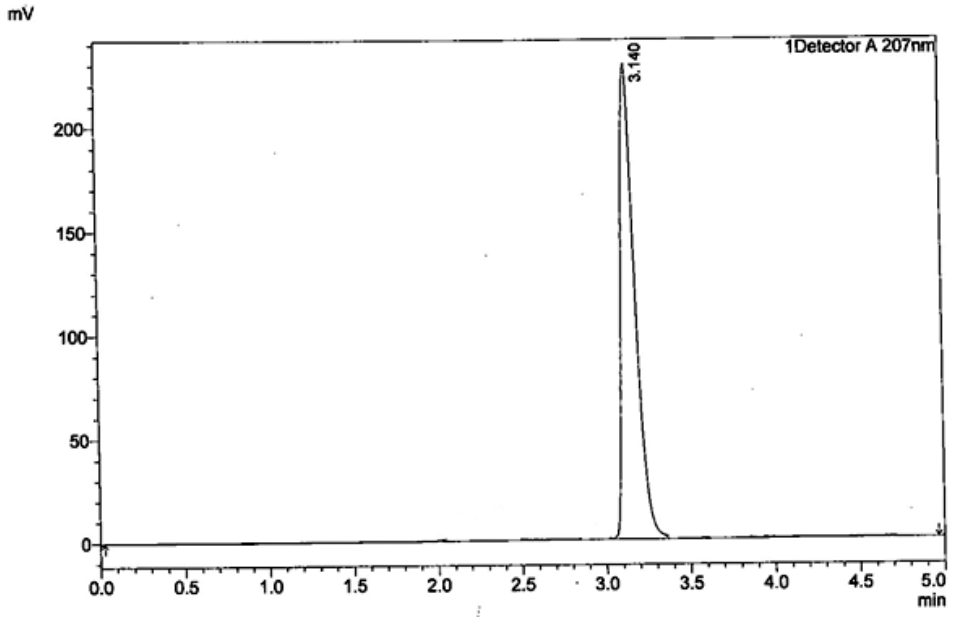
FIGURE – 21
REPEATABILITY ANALYSIS OF FORMULATION - 6
BY RP- HPLC

==== Shimadzu LabSolutions Data Image ====



Detector A 207nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.140	361435	229643	100.000		M
Total		361435	229643			

Name

E:\2012 - 13\2013-14\ALV\ALV-48.lcd

FIGURE –22
CHROMATOGRAM FOR RECOVERY ANALYSIS-1
(80%)

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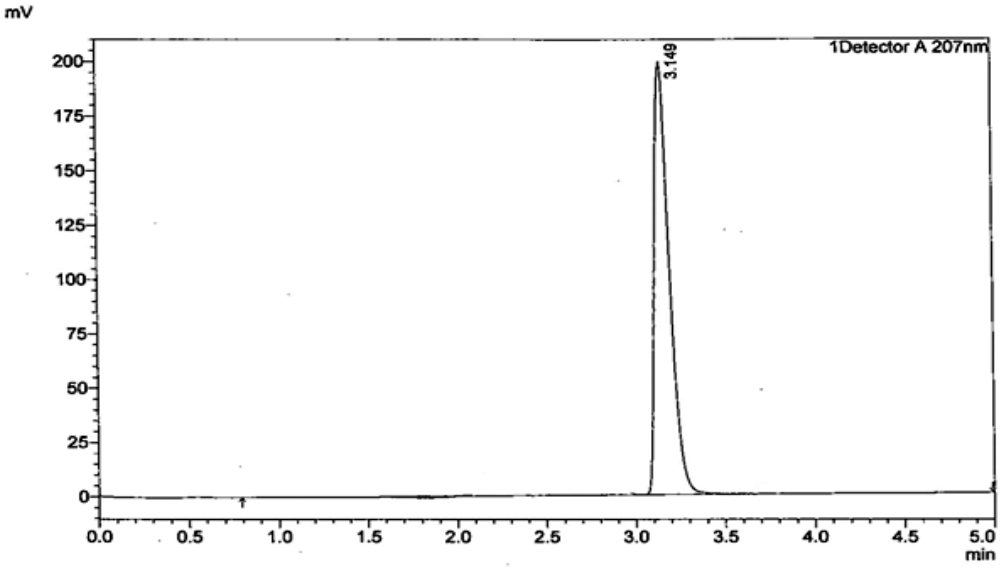
==== Shimadzu LabSolutions Data Image ====



Detector A 207nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.149	657532	199051	100.000		M
Total		657532	199051			

Name

E:\2012 - 13\2013-14\ALV\ALV-49.lcd

FIGURE – 23

CHROMATOGRAM FOR RECOVERY ANALYSIS - 2 (80%)

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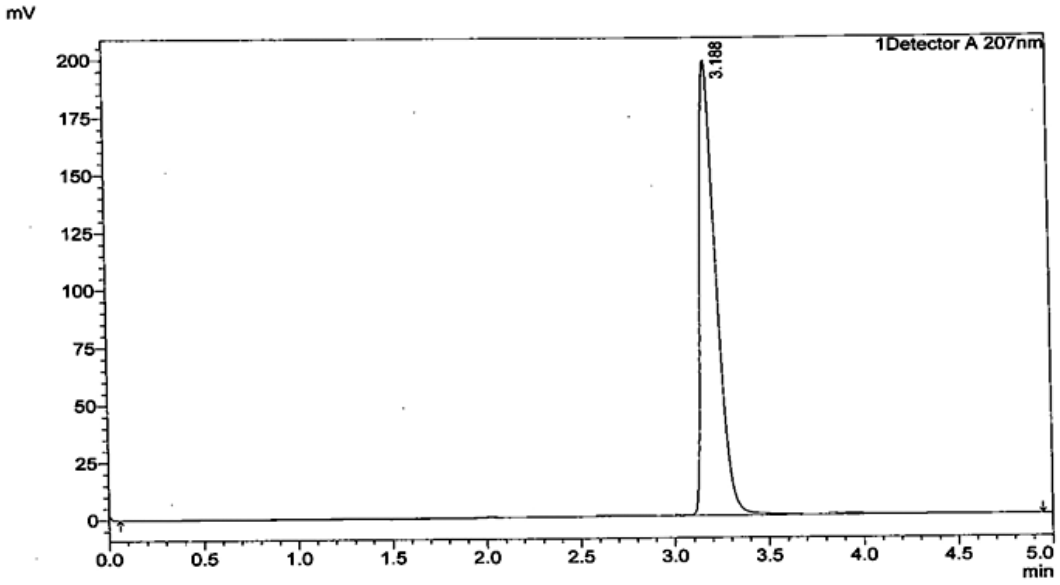
==== Shimadzu LabSolutions Data Image ====



Detector A 207nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.188	656321	198316	100.000		M
Total		656321	198316			

Name

E:\2012 - 13\2013-14\ALV\ALV-50.lcd

FIGURE – 24
CHROMATOGRAM FOR RECOVERY ANALYSIS - 3
(80%)

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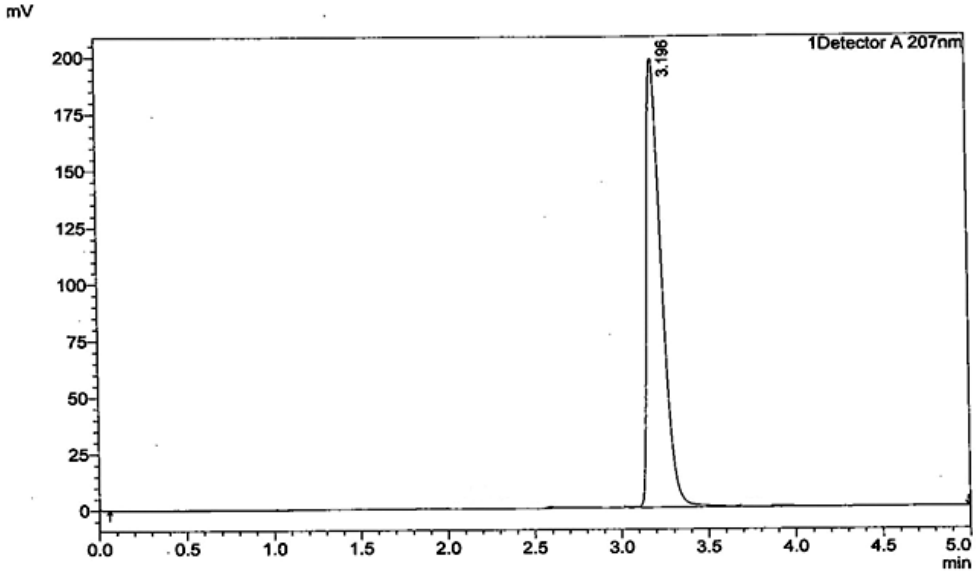
==== Shimadzu LabSolutions Data Image ====



Detector A 207nm

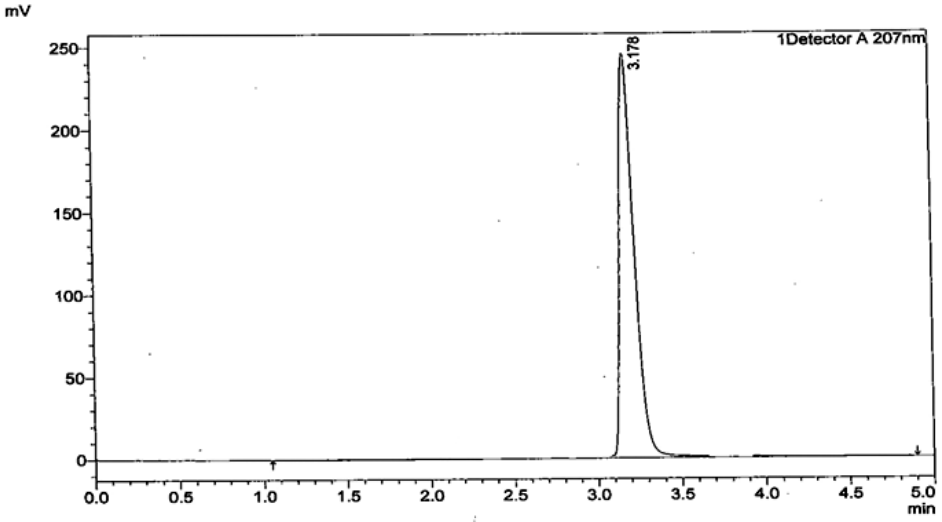
Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.196	653245	198278	100.000		M
Total		653245	198278			

Name

E:\2012 - 13\2013-14\ALV\ALV-51.lcd

FIGURE – 25
CHROMATOGRAM FOR RECOVERY ANALYSIS - 1 (100%)

==== Shimadzu LabSolutions Data Image ====



Detector A 207nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.178	732546	245668	100.000		M
Total		732546	245668			

Name

E:\2012 - 13\2013-14\ALV\ALV-52.lcd

FIGURE –26

CHROMATOGRAM FOR RECOVERY ANALYSIS - 2 (100%)

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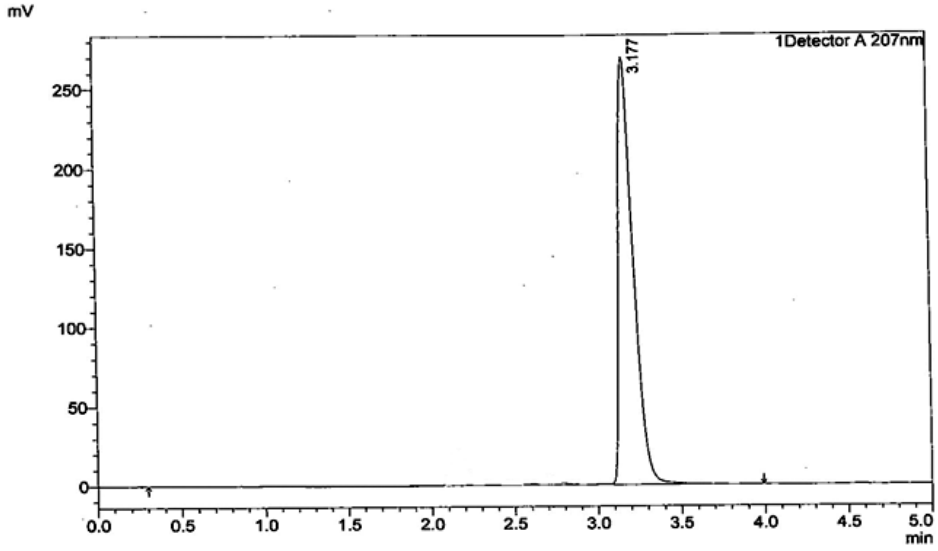
==== Shimadzu LabSolutions Data Image ====



Detector A 207nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.177	727254	270458	100.000		M
Total		727254	270458			

Name

E:\2012 - 13\2013-14\ALV\ALV-53.lcd

FIGURE – 27
CHROMATOGRAM FOR RECOVERY ANALYSIS - 3
(100%)

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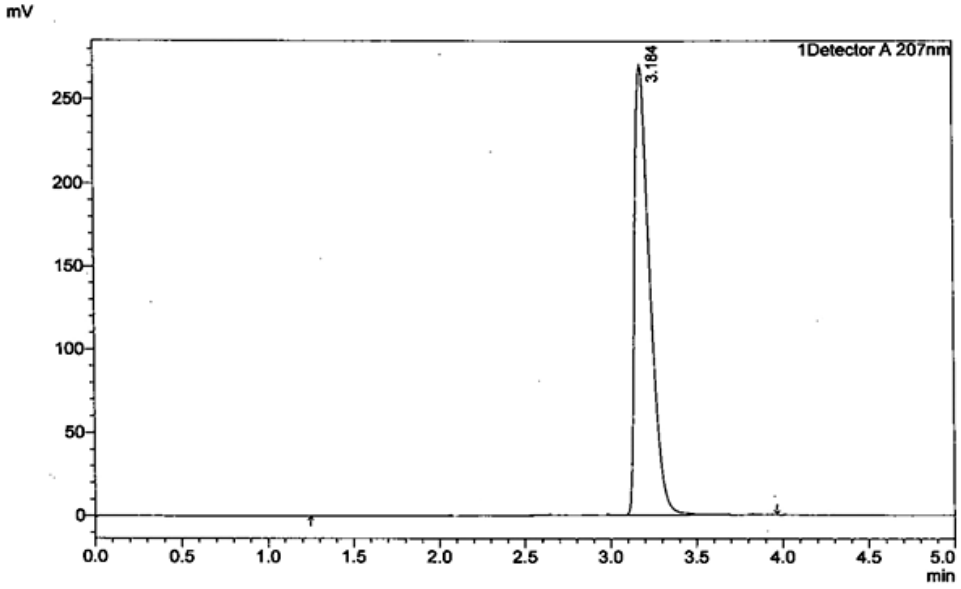
==== Shimadzu LabSolutions Data Image ====



Detector A 207nm

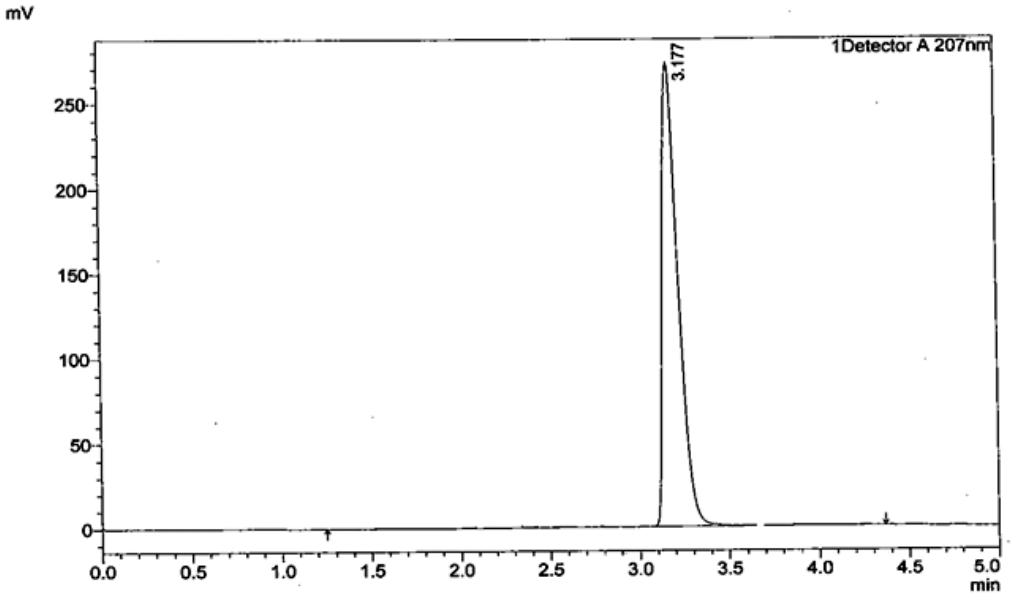
Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.184	801423	270937	100.000		M
Tota		801423	270937			

Name

E:\2012 - 13\2013-14\ALV\ALV-54.lcd

FIGURE-28
CHROMATOGRAM FOR RECOVERY ANALYSIS - 1
(120%)

==== Shimadzu LabSolutions Data Image ====



Detector A 207nm

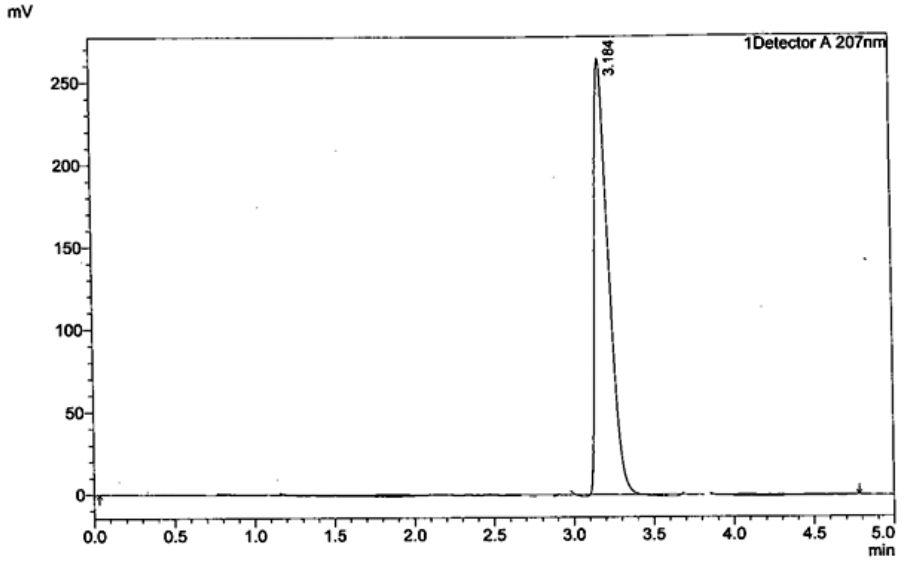
Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.177	729356	273343	100.000		M
Total		729356	273343			

Name

FIGURE – 29
CHROMATOGRAM FOR RECOVERY ANALYSIS - 2 (120%)

1/30/2014 3:14:03 PM Page 1 / 1

==== Shimadzu LabSolutions Data Image ====



Detector A 207nm

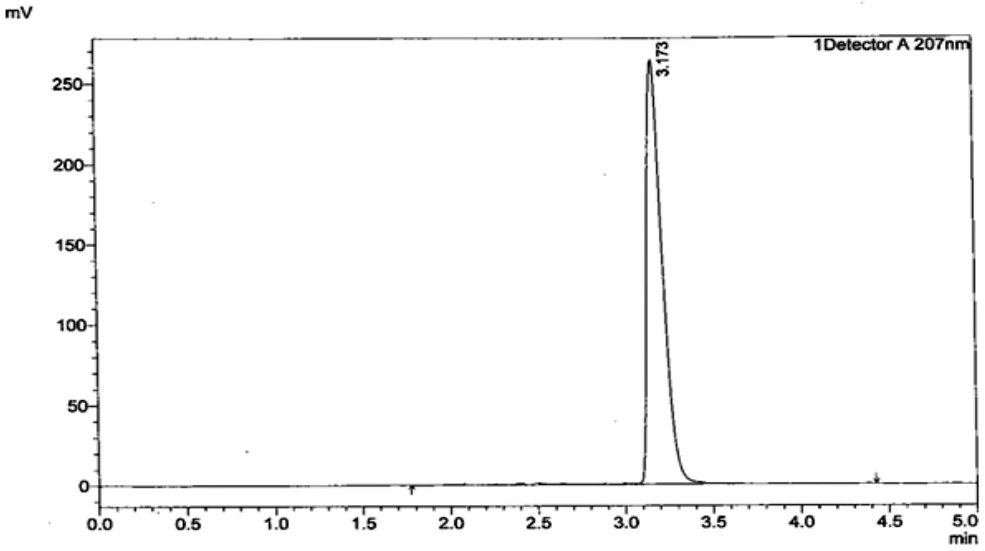
Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.184	800567	264681	100.000		M
Total		800567	264681			

Name

E:\2012 - 13\2013-14\ALV\ALV-59.lcd

FIGURE – 30
CHROMATOGRAM FOR RECOVERY ANALYSIS - 3
(120%)

==== Shimadzu LabSolutions Data Image ====



Detector A 207nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	3.173	800567	264495	100.000		M
Total		800567	264495			

Name

E:\2012 - 13\2013-14\ALV\ALV-60.lcd

TABLES

TABLE – 1
SOLUBILITY PROFILE OF ALVERINE CITRATE IN POLAR AND NON-POLAR SOLVENTS

S. No	SOLVENTS	EXTENT OF SOLUBILITY	CATEGORY
1.	Distilled water	10 mg in 20ml	Very slightly soluble
2.	Methanol	10 mg in 18µl	Freely soluble
3.	Ethanol	10 mg in 16 µl	Freely soluble
4.	Acetonitrile	10 mg in 30 µl	Freely soluble
5.	Di methyl Formamide	10 mg in 16µl	Freely soluble
6.	Acetone	10 mg in 8µl	Freely soluble
7.	Glacial Acetic acid	10 mg in 17 µl	Freely soluble
8.	Ethyl acetate	10 mg in 16 µl	Freely soluble
9.	Chloroform	10 mg in 24 µl	Freely soluble
10.	Diethyl ether	10 mg in 25 ml	Slightly soluble
11.	0.1M Hydrochloric Acid	10 mg in 17µl	Freely soluble
12.	0.1M Sodium Hydroxide	10 mg in more than 100 ml	Practically insoluble soluble
13.	Phosphate Buffer pH 7	10 mg in 350 µl	Sparingly soluble
14	Iso propyl alcohol	10 mg in 24 µl	Freely soluble
15	Carbon tetra chloride	10 mg in 6ml	Slightly soluble
17	Benzene	10 mg in more than 100 ml	Practically insoluble
18	Toluene	10 mg in more than 100 ml	Practically insoluble
19	Acid Phthalate Buffer (pH3)	10 mg in 90 ml	Very slightly soluble

20	Acid Phthalate Buffer (pH 2.2)	10 mg in 50ml	Very slightly soluble
21	Neutralized Phthalate Buffer (pH5)	10 mg in more than 100 ml	Practically insoluble
22	Alkaline Borate Buffer	10 mg in16 µl	Freely soluble
23	Dichloride methane	10mg in 1 ml	Sparingly soluble
24	Diethyl ether	10 mg in 25 ml	Slightly soluble
25	Phosphate buffer	10 mg in 350 µl	Sparingly soluble

TABLE – 2
OPTICAL CHARACTERISTICS OF ALVERINE CITRATE BY UV METHOD

PARAMETERS	VALUES*
λmax (nm)	207
Beer's law limit (μg/ ml)	4-24
Sandell's sensitivity ($\mu g/cm^2$/0.001 A.U)	0.0295
Molar absorptivity ($L\ mol^{-1}\ cm^{-1}$)	1.6204×10^{-4}
Correlation coefficient (r)	0.9995
Regression equation (Y= mx+c)	Y = 0.0339x+ 0.0029
Slope(m)	0.0339
Intercept(c)	0.0029
LOD (μg/ ml)	0.0632
LOQ (μg/ ml)	0.1915
Standard error	0.0103

*Mean of six observations

TABLE - 3
QUANTIFICATION OF RAW MATERIAL BY UV METHOD

S. No	Amount found (µg/ml)*	Percentage Obtained* (%)	Mean (%)	SD	%RSD	SE
1.	11.9933	99.99	100.12	0.0734	0.0734	0.0020
2 .	11.9964	99.97				
3.	12.0141	100.12				
4.	11.9993	99.99				
5.	11.9905	99.92				
6.	11.9905	99.92				

*Mean of six observations

TABLE - 4
QUANTIFICATION OF FORMULATION BY UV METHOD

Labeled amount (mg/tab)	Amount found (mg/tab)	Percentage Obtained* %(w/v)	% Mean	SD	% RSD	SE	CI
120.0000	119.5180	99.60	98.25	1.0034	1.0213	0.02789	96.60 to 99.90
120.0000	117.1350	97.61					
120.0000	116.4580	97.05					
120.0000	117.4850	97.90					
120.0000	117.3380	97.78					
120.0000	119.1980	99.33					

*Mean of six observations

TABLE - 5
INTRADAY ANALYSIS OF FORMULATION BY UV METHOD

Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
120.0000	117.4890	97.91	99.98	0.7434	0.7511	0.0465	97.76 to 100.20
120.0000	119.5220	99.60					
120.0000	118.1670	99.06					

* Mean of three observations

TABLE - 6
INTERDAY ANALYSIS OF FORMULATION BY UV METHOD

Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
120.0000	120.2880	100.24	99.54	0.9432	0.9475	0.1048	97.99 to 101.09
120.0000	119.9050	99.92					
120.000	118.1670	98.47					

*Mean of three observations

TABLE – 7
RUGGEDNESS STUDY BY UV METHOD
(DIFFERENT ANALYSTS)

Condition	Labeled Amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
Analyst 1	120.0000	119.9938	99.99	99.99	0.0734	0.0734	0.0020	99.87 to 100.11
	120.0000	119.9643	99.97					
	120.0000	120.1410	100.12					
	120.0000	119.9938	99.99					
	120.0000	119.9050	99.92					
	120.0000	119.9050	99.92					
Analyst 2	120.0000	121.4670	100.39	100.16	0.2352	0.2349	0.0065	99.77 to 100.55
	120.0000	120.2589	100.22					
	120.0000	120.5241	100.44					
	120.0000	120.1411	100.12					
	120.0000	119.8170	99.85					
	120.0000	119.9348	99.95					

*Mean of six observation

TABLE – 8
RUGGEDNESS STUDY BY UV METHOD
(DIFFERENT INSTRUMENTS)

Condition	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
Instrument I	120.0000 120.0000 120.0000 120.0000 120.0000 120.0000	119.9938 119.9643 120.1410 119.9938 119.9050 119.9050	99.99 99.97 100.12 99.99 99.92 99.92	98.25	0.0734	0.0734	0.0020	99.87 to 100.11
Instrument II	120.0000 120.0000 120.0000 120.0000 120.0000 120.0000	121.4670 120.2589 120.3149 121.4928 120.1116 119.9938	101.22 100.22 100.26 101.24 100.09 99.99	100.50	0.5710	0.5681	0.0159	99.50 to 101.44

*Mean of six observations

TABLE – 9
RECOVERY ANALYSIS OF FORMULATION BY UV METHOD

Amount present (µg/ml)	Amount added (µg/ml)	Amount found* (µg/ml)	Amount recovered (µg/ml)	% Recovery	SD	% RSD	SE	CI
9.7278	8.0000	17.9841	8.2563	103.20				
9.7278	8.0000	17.9598	8.2320	102.90				
9.7278	8.0000	17.9657	8.2379	102.97				
9.7278	10.0000	20.0341	10.3063	103.06				102.59
9.7278	10.0000	20.0194	10.2916	102.92	0.2251	0.2188	0.0028	to
9.7278	10.0000	20.0076	10.2798	102.80				103.09
9.7278	12.0000	22.0376	12.3098	102.58				
9.7278	12.0000	22.0406	12.3128	102.61				
9.7278	12.0000	22..0347	12.3069	102.56				
			Mean	102.84				

*Mean of three observations

TABLE – 10
SYSTEM SUITABILITY PARAMETERS FOR THE OPTIMIZED CHROMATOGRAM BY RP – HPLC METHOD

PARAMETERS	**VALUES**	**STANDARD LIMIT**
Retention time	3.143	----
Tailing factor	1.499	< 2
Asymmetrical factor	1.424	< 2
Number of Theoretical plates	6126	>2000
HETP	24.487	----
Capacity factor	1.03	>1

TABLE – 11
OPTICAL CHARACTERISTICS OF ALVERINE CITRATE BY RP-HPLC METHOD

PARAMETERS	VALUES*
Detection wavelength (nm)	207
Beer's law limit (µg/ml)	10 – 60
Correlation coefficient (r)	0.9999
Regressionequation (Y = mx + c)	Y = 18218.0726+ (-2465.75)
Slope (m)	18218.726
Intercept (c)s	-2465.75
LOD (ng/ µl)	0.0670
LOQ (ng/ µl)	0.2031
Standard error	4435.1146

*Mean of three observations

TABLE – 12
ANALYSIS OF FORMULATION BY RP- HPLC METHOD

Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Mean (%)	SD	% RSD	SE	CI
120.0000	119.8561	99.88	100.06	0.2186	0.2185	0.0061	99.70 to 100.42
120.0000	120.0577	100.01					
120.0000	120.2900	100.24					
120.0000	119.8993	99.92					
120.0000	120.5086	100.42					
120.0000	119.8928	99.91					

*Mean of six observations

TABLE –13
RECOVERY ANALYSIS OF FORMULATION
BY RP-HPLC METHOD

Amount present (µg/ml)	**Amount added (µg/ml)**	**Amount found* (µg/ml)**	**Amount recovered (µg/ml)**	**% Recovery**	**SD**	**% RSD**	**SE**	**CI**
20.0066	16.0000	36.2274	16.2208	101.38				
20.0066	16.0000	36.1611	16.1551	100.96				
20.0066	16.0000	35.9924	15.9858	99.91				
20.0066	20.0000	40.3452	20.3386	101.69				100.05
20.0066	20.0000	40.0547	20.0481	100.24	0.5736	0.5697	0.0071	to
20.0066	20.0000	40.1701	20.1641	100.82				101.33
20.0066	24.0000	44.1258	24.1192	100.50				
20.0066	24.0000	44.0789	24.0723	100.30				
20.0066	24.0000	44.1100	24.1034	100.43				
			Mean	100.69				

*Mean of three observations

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